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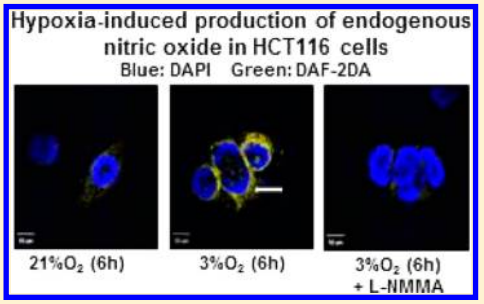
Nitric Oxide Produced Endogenously Is Responsible for Hypoxia-Induced HIF-1α Stabilization in Colon Carcinoma Cells

Rajdeep Chowdhury,[†] Luiz C. Godoy,[†] Apinya Thiantanawat,[‡] Laura J. Trudel,[†] William M. Deen,[§] and Gerald N. Wogan*[,†]

[†]Department of Biological Engineering and [§]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

[‡]Laboratory of Pharmacology, Chulabhorn Research Institute, Bangkok 10210, Thailand

ABSTRACT: Hypoxia-inducible factor-1α (HIF-1α) is a critical regulator of cellular responses to hypoxia. Under normoxic conditions, the cellular HIF-1α level is regulated by hydroxylation by prolyl hydroxylases (PHDs), ubiquitylation, and proteasomal degradation. During hypoxia, degradation decreases, and its intracellular level is increased. Exogenously administered nitric oxide (NO)-donor drugs stabilize HIF-1α; thus, NO is suggested to mimic hypoxia. However, the role of low levels of endogenously produced NO generated during hypoxia in HIF-1α stabilization has not been defined. Here, we demonstrate that NO and reactive oxygen species (ROS) produced endogenously by human colon carcinoma HCT116 cells are responsible for HIF-1α accumulation in hypoxia. The antioxidant *N*-acetyl-L-cysteine (NAC) and NO synthase inhibitor N^G-monomethyl L-arginine (L-NMMA) effectively reduced HIF-1α stabilization and decreased HIF-1α hydroxylation. These effects suggested that endogenous NO and ROS impaired PHD activity, which was confirmed by reversal of L-NMMA- and NAC-mediated effects in the presence of dimethyloxaloylglycine, a PHD inhibitor. Thiol reduction with dithiothreitol decreased HIF-1α stabilization in hypoxic cells, while dinitrochlorobenzene, which stabilizes *S*-nitrosothiols, favored its accumulation. This suggested that ROS- and NO-mediated HIF-1α stabilization involved S-nitrosation, which was confirmed by demonstrating increased S-nitrosation of PHD2 during hypoxia. Our results support a regulatory mechanism of HIF-1α during hypoxia in which endogenously generated NO and ROS promote inhibition of PHD2 activity, probably by its S-nitrosation.



INTRODUCTION

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that plays a key role in regulating cellular functions in response to variations in tissue oxygen tension. HIF-1α is continuously produced, but in normoxia, low cellular levels are maintained by a sequential process involving hydroxylation by prolyl hydroxylases (PHDs), binding to von Hippel-Lindau protein (VHL), ubiquitylation, and proteosomal degradation. When hypoxia develops, as in poorly vascularized solid tumors, HIF-1α levels are augmented, causing increased expression of HIF-1α-regulated genes that contribute to enhanced tumor cell growth, angiogenesis, and resistance to chemotherapeutic agents.[1,2]

On the basis of a comprehensive review of estimates from the literature, Thomas et al. proposed functional categories for nitric oxide (NO) concentrations, ranging from cGMP-mediated signaling processes at ~1−30 nM, modulation of kinase and transcription factor activity (e.g., Akt, HIF-1α, p53) at ~30−400 nM, and pathological nitrosative and oxidative stresses above ~500−1000 nM.[3] Accurate evidence for concentration-dependent biological functions of NO requires controlled delivery of predictable and biologically relevant steady-state levels of NO and O_2 to cultured cells. NO can be introduced into cell cultures by several methods. Frequently used are "NONOates" that release NO with various kinetics to provide transient, nonuniform levels of NO that must be averaged over time to define the exposure level. In addition to the absence of defined, steady-state levels of NO, interpretation of data obtained from NONOates is complicated by unknown effects of non-NO free radical species generated along with NO, generation of alternative reactive nitrogen species in addition to NO, and the need for several different NO donor compounds to span the range of physiologically relevant NO concentrations.

Increased levels of HIF-1α protein have been associated with exposure to exogenous NO and reactive oxygen species (ROS) in various cell types and tissues.[4−8] Pharmacological and genetic manipulations that increase in vitro ROS and NO production have been shown to influence oxygen sensing, PHD activity, HIF-1α interaction with VHL, and hence HIF-1α stability.[4,5,9,10] In relating various cellular responses to NO exposures, Thomas et al.[3] emphasize the importance of local concentration and duration as determinants of responses such as HIF stabilization. Stabilization was estimated to occur between 100 and 300 nM, based on external exposure of cells

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to NONOates or to coculture with activated macrophages. They noted, however, that uncertainty exists regarding quantitative features of dose−response relationships induced by these types of exogenous and endogenous exposures to NO.

Exogenous NO exposure has been postulated to mimic hypoxia under given experimental conditions.[9,11,12] Exogenous NO-mediated HIF-1α modification by in vitro S-nitrosation has been shown to stabilize the protein,[13] and regulation of PHD activity by S-nitrosation has also been proposed,[8,14] although the latter has yet to be confirmed experimentally. Thus, the potential regulatory role of relatively low levels of endogenously produced NO in this context remains undefined. In this study, we sought to address this issue in experiments focused on HIF stabilization and related responses in HCT116 colon carcinoma cells. Endogenous exposure was accomplished in cells grown in atmospheres containing 21 or 3% oxygen, whereas external exposure was achieved through controlled steady-state delivery of NO with a reactor specifically designed for this purpose. We evaluated the contributions of endogenously produced NO and ROS to HIF-1α stabilization during hypoxia and found among other effects S-nitrosation of PHD2, which was in turn associated with lower HIF-1α hydroxylation and a subsequent decrease in degradation of the transcription factor.

MATERIALS AND METHODS

Cell Culture, NO Exposure, and Hypoxia. HCT116 human colon carcinoma cells (a gift from C. C. Harris, NCI, Bethesda, MD) were cultured at 37 °C, 5% CO_2, in McCoy's 5A medium containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cells at a density of 70−80% confluence were plated in 60 mm dishes 24 h before exposure to hypoxia or NO. Using an NO delivery system previously described,[15,16] cells were exposed to steady-state concentration of 1.74 μM NO for the indicated times, resulting in specific cumulative doses. Cells exposed to argon, the carrier gas for NO, served as the control.

For hypoxia treatment, 3×10^6 cells in 3 mL of fresh medium were placed in an airtight Modular Incubator Chamber (Billups-Rothenberg, Inc.), flushed with gas comprising 3% O_2, 5% CO_2, and 92% N_2 for 3 min, and kept in an incubator at 37 °C for indicated time periods. Control cells were placed in a similar chamber purged with 21% O_2, 5% CO_2, and 74% N_2. Cell culture reagents were purchased from Lonza, and gases were from Airgas.

Treatments. Cells were plated overnight in 6 cm dishes and treated for 30 min prior to hypoxia exposure, as required with specific compounds: NOS inhibitors, N^G-monomethyl L-arginine (L-NMMA, 4 mM) (Calbiochem), and nitro-L-arginine (NNA, 10 μM); NO scavenger, 2-4-carboxyphenyl-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide (PTIO, 300 μM); antioxidant, *N*-acetyl-L-cysteine (NAC, 5 mM); proteasome inhibitor, MG132 (10 μM); competitive PHD inhibitor, dimethyloxaloylglycine (DMOG, 1 mM); and thiol-reductant, dithiothreitol (DTT, 0.5−1.5 mM). In some specific experiments, cells were exposed to selective inducible nitric oxide synthase (iNOS) inhibitor, *N*-[(3-aminomethyl)benzyl]-acetamidine (1400W, 1 mM) (Cayman Chem., Ann Arbor, MI), overnight prior to hypoxia exposure. Dichloronitrobenzene (DNCB, 60 μM), which stabilizes cellular *S*-nitrosothiols, was applied to cultured cells for 60 min before hypoxia treatment. Chemicals were from Sigma unless otherwise stated.

Cell Viability Analysis. Live cells were identified by trypan blue exclusion, which produced results comparable to plating efficiency, and MTT assays were performed in our lab.[17,18]

Detection of ROS and NO. The generation of ROS was quantified using the fluorescent probe 5-(or-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA, Invitrogen) as follows. Cells were grown in black, clear-bottom 96-well microplates (1×10^5 cells/well) for 18 h, loaded with new media with or without inhibitors, and exposed to hypoxia. Next, cells were incubated at 37 °C in serum- and antibiotic-free medium containing 10 μM CM-H2DCFDA. After the cells were washed twice with PBS, the fluorescence intensity was measured in an automatic plate reader (Spectra Max Gemini, Molecular Device).

For the detection of NO by microscopy, after treatments, cells were incubated with the NO-specific fluorescent dye diaminofluorescein-2 diacetate (DAF-2DA) following the manufacturer's instructions (Cell Technology, Inc.). Cells were then mounted in Ultra Cruz mounting media with DAPI (Santa Cruz Biotechnology) and analyzed in a Zeiss LSM confocal microscope.

The NO_2^- concentration in cell culture medium was measured by analysis with a Sievers Nitric Oxide Analyzer (NOA 280i) from GE Analytical Instruments, using the manufacturer's protocol for liquid sample analysis, briefly summarized as follows. NO is detected by a gas-phase chemiluminescence reaction of NO with ozone. Certain related species can be converted to NO in the purge vessel of the analyzer, and the released NO is carried by argon to the detector along with any preformed NO. A solution of acetic acid containing 50 mM potassium iodide was used in the purge vessel, as recommended by the manufacturer for detection of NO_2^- (and not NO_3^- or GSNO). Potential signal contributions derived from species other than NO_2^- were assessed by parallel analysis of samples treated with sulfanilamide, which specifically scavenges NO_2^-.[19] This treatment completely eliminated the signal from our samples, confirming that what was being measured was NO_2^-, as assumed when calculating the NO concentration in the medium (see below). Nitrite concentrations were calculated based on a standard curve generated with $NaNO_2$.

Immunofluorescence Staining. Monolayers of cells (1×10^5) were grown on coverslips, exposed to hypoxia, fixed with methanol at −20 °C for 30 min, and rehydrated with PBS. Nonspecific binding sites were blocked with 5% nonfat dry milk for 1 h, followed by overnight incubation with primary antibody (anti-HIF-1α; BD Transduction Laboratories) diluted 1:100. After three PBS washes, cells were incubated with Texas Red-conjugated secondary antibody (TR, Santa Cruz Biotechnology) diluted 1:200 for 1 h. Finally, cells were washed with PBS and mounted in mounting medium and analyzed in a Nikon Eclipse E600 fluorescence microscope.

Transient Transfection and Luciferase Assay. Prior to treatment, HCT116 cells were seeded at a density of 2×10^5 cells/well in a six-well plate, incubated overnight, and then transfected using Lipofectamine Reagent (Invitrogen) with plasmid DNA (0.5 μg). Inducible HIF-1α-responsive firefly luciferase reporters construct and constitutively expressing *Renilla* luciferase construct (SABiosciences) were transfected for luciferase assays. Eighteen hours after transfection, cells were exposed to hypoxia with or without inhibitors for specific time periods, and the luciferase activity was measured in a luminometer (Spectra Max Gemini, Molecular Devices) using Dual Luciferase Reporter Assay System (Promega).

siRNA-Mediated Knockdown. HCT116 cells seeded at a density of 2×10^5 cells/well were allowed to grow for 18 h in antibiotic-free medium containing FBS, washed, and replenished with medium. They were then transfected with 60 pmol of siRNA duplex corresponding to HIF-1α following the manufacturer's instructions (Santa Cruz Biotechnology) and incubated at 37 °C for 24 h. They were then exposed to hypoxia for the stipulated time periods and subjected to Western blotting as described below.

Immunoblotting. Western blot analyses were performed as described previously.[18] Briefly, cells were lysed in RIPA buffer (Sigma), and the protein content was measured by BCA Assay (Thermo Scientific). Samples were mixed with an equal volume of Laemmli Sample Buffer (Bio-Rad), heat-denatured (100 °C, 10 min) with β-mercaptoethanol (β-ME) Sigma, loaded into precast SDS-PAGE gels (Bio-Rad), transferred to nitrocellulose membranes, and probed with specific antibodies. Cytoplasmic and nuclear fractions were obtained using a nuclear extraction kit (Millipore) following the manufacturer's instructions. Antibodies used were as follows: anti-hydroxy-HIF-1α (Pro564) (Cell Signaling Technology), anti-Histone H1 (Santa Cruz Biotechnology), anti-PHD2 (Abcam), and anti-HIF-1α (BD Transduction Laboratories). Secondary antibodies were

horseradish peroxide-conjugated goat antirabbit IgG or rabbit antimouse IgG or rabbit antigoat IgG (Santa Cruz Biotechnology). As a protein loading control, membranes were stripped and reprobed with antimouse β-actin monoclonal antibody (Sigma). HIF-1α detection by Western blot was done by processing lysates in less than 3 min to prevent degradation upon exposure to room air.

S-Nitrosation. S-Nitrosation was assessed by the biotin switch assay,[15,20] with modifications. Cells exposed to hypoxia (2 or 4 h) or exogenous NO (1.8 or 3.6 h) were washed in HEN buffer (250 mM HEPES sodium salt, pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine) and lysed for 30 min at 4 °C in HEN buffer containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.1% SDS, and protease inhibitors. Lysates were centrifuged at 14000*g*, and 1 mg of total protein from each sample was precipitated with cold acetone and resuspended in HEN buffer containing 1% SDS (HENS) at a final protein concentration of 0.8 μg/μL. Free thiols were blocked by addition of 20 mM (final concentration) of (*S*)-methyl methanethiosulfonate (MMTS) and incubation at 50 °C for 30 min with periodic vortexing, followed by another acetone precipitation and resuspension in 200 μL of HENS buffer. Biotinylation of *S*-nitrosothiols was carried out by incubation with 20 mM ascorbic acid and 1 mM *N*-(6-(biotinamido)hexyl)-3-(2-pyridyldithio)-propionamide (biotin-HPDP, Pierce-Thermo Scientific) for 1 h at room temperature with gentle mixing. To verify specific labeling of nitrosothiols, an internal control was performed in which samples were not treated with ascorbic acid, which displaces NO from *S*-nitrosothiols, allowing biotin to bind to nascent thiol groups. Excess biotin-HPDP was removed by acetone precipitation, and samples were resuspended in 300 μL of HENS buffer plus 600 μL of neutralization buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100). Biotinylated proteins were isolated by overnight incubation with neutravidin-coupled agarose beads (Pierce-Thermo Scientific). Beads were then washed with neutralization buffer containing 600 mM NaCl. Proteins were recovered from beads by addition of equal parts of elution buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 100 mM β-ME) and reducing SDS–PAGE sample buffer, followed by heating at 100 °C (10 min). S-Nitrosation of PHD2 in these samples was verified by immunoblotting with anti-PHD2 antibody (Abcam). To confirm the role of S-nitrosation in stabilization of HIF-1α, HCT116 cells were treated with DTT, used as a thiol-reductant or DNCB, which favors stabilization of cellular nitrosothiols, and analyzed for abundance of HIF-1α protein.

Calculation of NO Concentrations. Endogenous NO concentrations were determined from observed rates of NO_2^- accumulation by using the known kinetics of NO autoxidation, which are second-order in NO and first-order in O_2.[21] If the concentrations were spatially uniform, calculating the NO concentration from the measured rate of NO_2^- formation and known O_2 concentration would be straightforward. As discussed in the Appendix, it was inferred that the O_2 concentration in the culture medium was indeed nearly uniform. However, the NO concentration must have decreased rapidly with height (z), ranging from a maximum at the level of the cells ($z = 0$) to nearly zero at the gas–liquid interface ($z = L$). Accordingly, a reaction–diffusion model was needed to calculate the position-dependent NO concentration, $C_{NO}(z)$. The model describes the competition between autoxidation and diffusional loss of NO from the system and accounts for the variation in the local NO_2^- formation rate with z. For our particular experimental conditions, the NO concentration at the cells is given by

$$C_{NO}(0) = \left(\frac{3R}{4kC_0}\right)^{1/2} \quad (1)$$

where R is the observed rate of NO_2^- accumulation (the rate averaged over the entire volume of medium), k is the rate constant for autoxidation, and C_0 is the aqueous O_2 concentration. As discussed in the Appendix, this simple expression should be accurate to within a few percent. The parameter values used are listed there.

Statistical Analysis. All experiments were repeated 3–4 times. Statistical analysis was performed using a two-tailed Student's t test, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Hypoxia-Induced HIF-1α Accumulation. Upon oxygen deprivation of cells, HIF-1α is stabilized and translocates to the nucleus where it transcriptionally activates genes whose products support cell survival and growth.[1,2] To establish conditions to be employed in our experiments, we first compared effects of duration of exposure to hypoxia (3% O_2) or 21% O_2 on cell growth; no significant reduction of live cell counts was observed until 16 h of hypoxia, after which viability decreased. In further experiments, we therefore exposed cells to hypoxia for shorter periods; as shown in Figure 1A,B, exposure for 2–6 h induced a gradual increase in HIF-1α protein level, as well as its translocation into the nucleus.

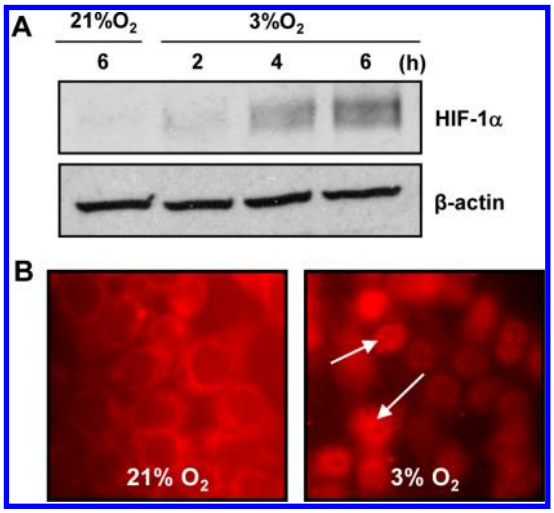

Figure 1. Hypoxia induces HIF-1α accumulation in HCT116 cells. (A) HIF-1α protein accumulation following exposure of cells to 3% O_2. (B) Nuclear localization of HIF-1α visualized through immunofluorescent staining; arrows indicate HIF-1α localized in the nucleus of cells exposed to 3% O_2 for 6 h.

Generation of ROS and NO during Hypoxia. It has been proposed that ROS generation is a prerequisite for HIF-1α stabilization under hypoxia.[5,10] We detected a significant increase in intracellular ROS content upon exposure of cells to hypoxia for 4–6 h (Figure 2A). Mitochondria were identified as the source of ROS (data not shown), and ROS levels were significantly attenuated by the antioxidant NAC (Figure 2A). Endogenous cellular NO concentrations were calculated from the rates of NO_2^- accumulation using eq 1. For the controls (21% O_2), there was not a significant increase in NO_2^- concentration over time (Figure 2B). Accordingly, the NO concentration was undetectable. The progressively increasing NO_2^- concentrations for hypoxic cells (3% O_2, Figure 2B) gave average NO_2^- accumulation rates (R) of 14 pM/s for the 2–4 h period and 7.6 pM/s for the 4–6 h period. The corresponding NO concentrations from eq 1 were 0.36 and 0.27 μM (Figure 2B). Thus, for the hypoxic cells, the NO concentration remained at about 0.3 μM for the entire period of observation, whereas for control cells, it was evidently near zero. To further characterize the ability of these cells to produce NO, we assessed levels of iNOS by immunoblotting. As shown in Figure 2C, a significant increase in iNOS expression was induced

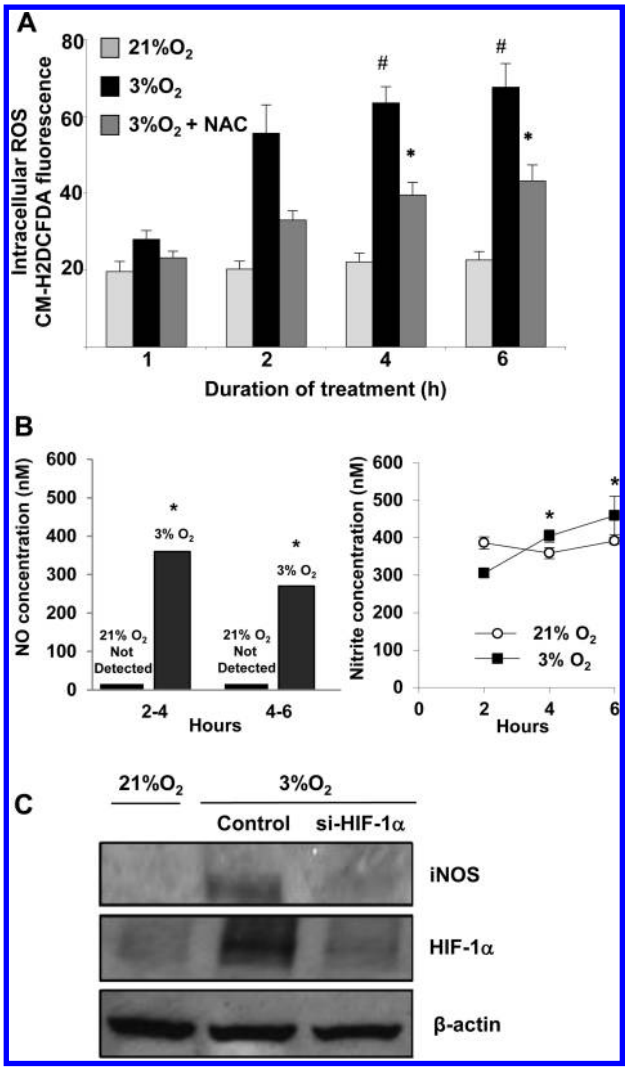

Figure 2. Hypoxia induces endogenous ROS and NO generation in HCT116 cells. (A) ROS levels measured by corrected CM-H2DCFDA fluorescence intensity in cells exposed to 21% O_2, 3% O_2, or 3% O_2 in the presence of antioxidant (NAC). The symbols # and * indicate significant differences from respective controls (4 h hypoxia vs control, $P < 0.00007$; 6 h hypoxia vs control, $P < 0.00004$; and 4 h hypoxia vs NAC, $P < 0.00006$). (B) Effect of hypoxia on endogenous production of NO by HCT116 cells, determined by analysis of culture medium with Sievers nitric oxide analyzer (NOA). The graph on the right shows actual nitrite concentration measured by NOA over time. The symbol * indicates a statistically significant ($P < 0.05$) increase in nitrite concentrations at 4 and 6 h over time as compared to 2 h at 3% O_2. (C) Changes in levels of iNOS and HIF-1α protein (Western blot) caused by exposure of cells to 3% O_2 (6 h) and knockdown with siRNA specific for HIF-1α.

during exposure of cells to hypoxia for 6 h. Moreover, the existence of a positive HIF-1α-iNOS feedback loop is evident from the data, which shows that silencing of HIF-1α by si-RNA led to concomitant reduction in iNOS protein levels, suggesting that iNOS may be under transcriptional control of HIF-1α.

Endogenous ROS and NO Sustain HIF-1α Accumulation. The HIF-1α content was analyzed in hypoxic cells pretreated with NOS inhibitors (L-NMMA and NNA), an NO scavenger (PTIO), an antioxidant (NAC), or a combination of L-NMMA and NAC (Figure 3A). While all treatments resulted in diminished stabilization of HIF-1α, the combination of L-NMMA and NAC was most effective in preventing its

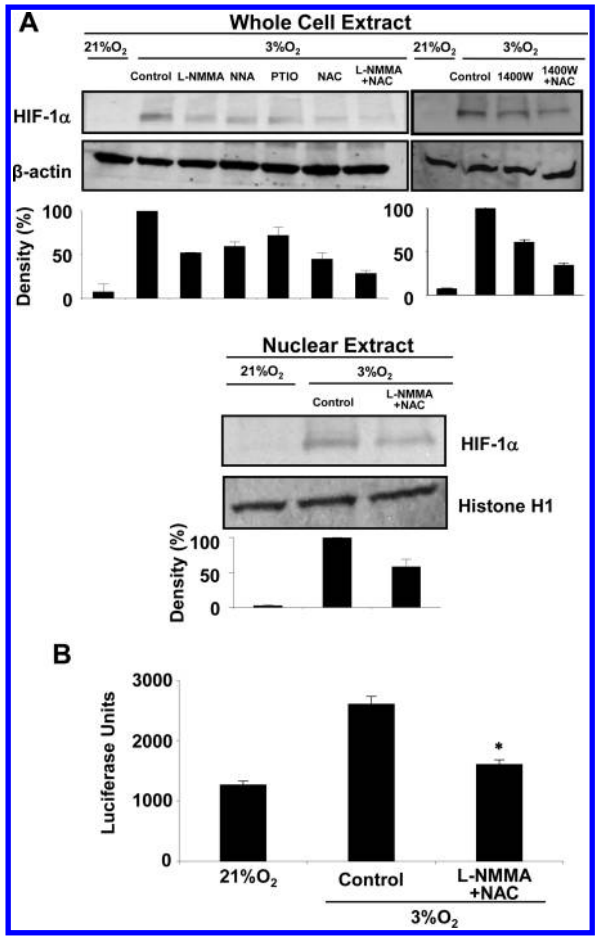

Figure 3. ROS and NO induce hypoxic HIF-1α accumulation and increased transcriptional activity. (A) Cells were exposed to 3% O_2 or 3% O_2 plus NOS inhibitor and/or antioxidant and analyzed for HIF-1α protein in whole cell and nuclear extracts by Western blot. β-Actin and histone H1 served as loading controls for whole cell and nuclear extract, respectively. (B) NOS inhibitor and antioxidant treatment attenuates 3% O_2, 6 h-induced expression of HIF-1α-dependent promoter luciferase construct. The symbol * indicates significantly different values in inhibitor treated samples as compared to 3% O_2, $P < 0.01$.

accumulation in whole cell extract as well as in the nucleus (Figure 3A). When treated with the iNOS-specific inhibitor 1400W, cells showed similar results, with enhanced degradation of HIF-1α in the presence of 1400W and NAC as compared to iNOS inhibitor alone (Figure 3A). Furthermore, cells transfected with a HIF-1α-dependent luciferase reporter construct showed marked reduction in luciferase activity following L-NMMA and NAC treatment and hypoxia, indicating decreased HIF-1α transcriptional activity (Figure 3B). Semiquantitative RT-PCR showed no change in transcription of the HIF-1α gene under these conditions (data not shown), suggesting that the observed decrease in protein depended on post-transcriptional mechanisms.

Hypoxia-Induced Endogenous ROS and NO Impair PHD Activity. During hypoxia, the PHD activity is decreased, resulting in HIF-1α accumulation.[1,2] To assess contributions of NO and ROS to this process, we analyzed levels of hydroxylated HIF-1α as a measure of PHD activity after treatment of cells with L-NMMA and NAC. The proteasomal inhibitor MG132 was used to decrease degradation and facilitate detection of hydroxylated HIF-1α. As shown in

Figure 4A, higher levels of hydroxylated HIF-1α were found in hypoxic cells treated with L-NMMA and NAC than in

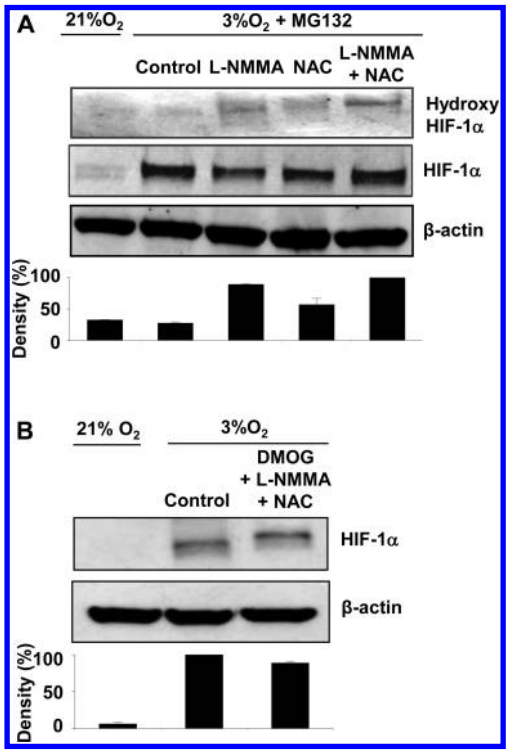

Figure 4. Hypoxia-induced ROS and NO impair PHD2 activity. (A) Levels of hydroxylated HIF-1α were analyzed by Western blot using anti-hydroxy (Pro564)-HIF-1α antibody in cells exposed to L-NMMA and/or NAC during exposure to 3% O_2 for 6 h. The proteasome inhibitor MG132 was added to enhance detection of hydroxy HIF-1α. (B) Cells were treated with PHD inhibitor DMOG in the presence/absence of L-NMMA and NAC during 6 h of exposure to 3% O_2. Protein extracts were analyzed for HIF-1α by Western blot.

untreated hypoxic cells. These results suggest that NO and ROS lead to increased HIF-1α stabilization in hypoxia by inhibiting HIF-1α hydroxylation, presumably reflecting decreased proline hydroxylase function.

Because PHD expression remained unaltered under the above experimental conditions (data not shown), we sought to assess the mechanism through which NO and ROS decrease PHD activity, leading to reduced HIF-1α hydroxylation. PHDs have several essential cofactors, such as 2-oxoglutarate, and the oxoglutarate analogue DMOG constitutes a potent inhibitor of PHD activity.[22] As shown in Figure 3A, L-NMMA and NAC treatment reduced HIF-1α stabilization during hypoxia. On the other hand, when PHD was inhibited by DMOG, L-NMMA and NAC failed to reduce HIF-1α stabilization (Figure 4B). These data indicate that L-NMMA and NAC effects depend on PHD activity and, therefore, that PHD activity may be the critical element that NO and ROS regulate during hypoxia.

Hypoxia Induces S-Nitrosation of PHD2. NO and ROS react to form reactive nitrogen species (RNS),[3,23,24] a major consequence of which can be the post-translational modification of proteins by S-nitrosation.[25] S-Nitrosation of PHDs has been demonstrated in cell-free in vitro systems, but its occurrence and implications in vivo remain uncertain.[26] In our experiments, treatment of intact hypoxic cells with the thiol-reducing agent DTT resulted in lower stabilization of HIF-1α (Figure 5A). On the other hand, when cells were

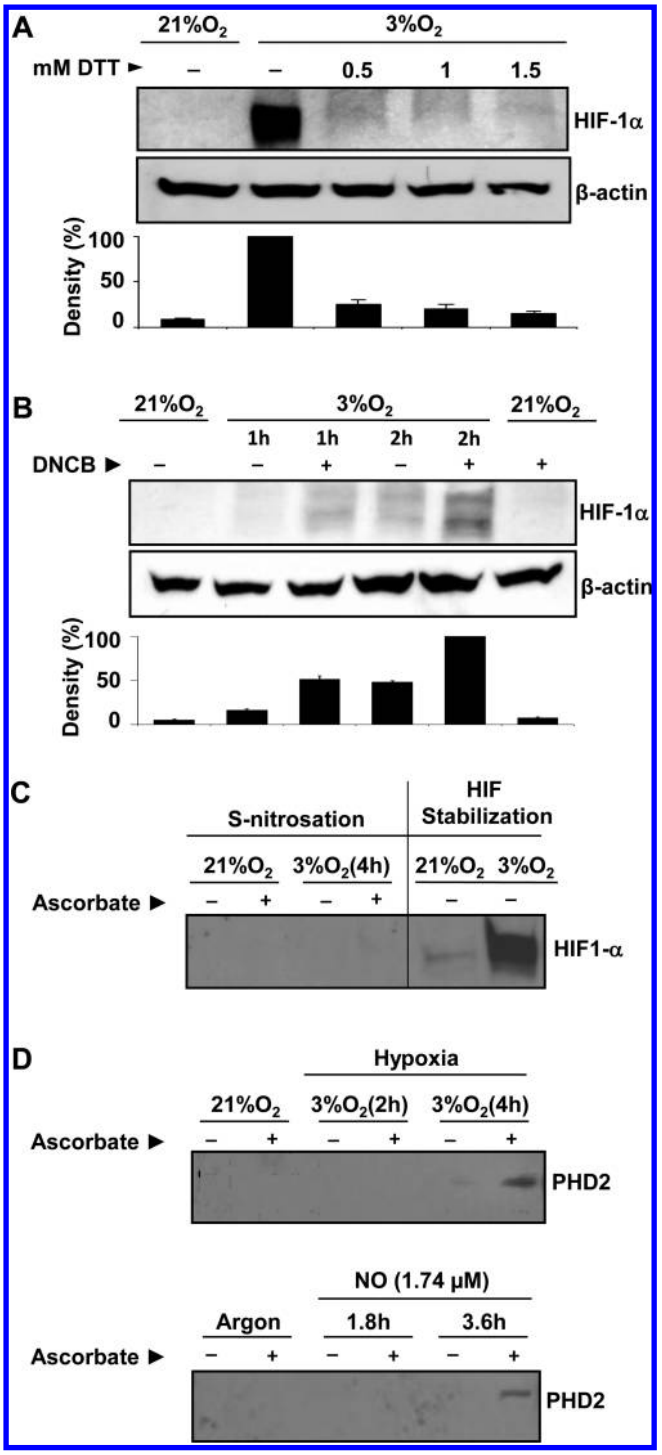

Figure 5. Evidence for S-nitrosation of PHD2. (A) Hypoxia-induced HIF-1α accumulation was blocked by treatment with 0−1.5 mM DTT to destabilize nitrosothiols during exposure of HCT116 cells to 3% O_2 for 4 h. (B) Hypoxia-induced HIF-1α accumulation was restored by treatment with DNCB, which stabilizes cellular nitrosothiols, during exposure to 3% O_2 for 1 or 2 h. (C) HIF-1α protein was stabilized by exposure to 3% O_2 for 4 h but was not S-nitrosated, as determined by biotin switch assay. (D) PHD2 S-nitrosation. The biotin switch technique was used to verify PHD2 S-nitrosation following exposure to exogenous NO or 3% O_2. Argon served as a negative control. To verify specific labeling of nitrosothiols, an internal control was performed in which samples were not treated with ascorbic acid, which displaces NO from nitrosothiols, allowing biotin to bind to nascent thiols.

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treated with DNCB, which favors stabilization of cellular *S*-nitrosothiols, increased HIF-1α accumulation was seen when compared to cells exposed to hypoxia alone (Figure 5B). These results led us to hypothesize that thiol-sensitive mechanisms such as S-nitrosation are involved in HIF-1α stabilization.

Accordingly, we analyzed S-nitrosation levels in the HIF-1α protein and also in PHD2, the most abundant PHD, in cells exposed to hypoxia or to exogenous NO. Exposure of cells to hypoxia resulted in accumulation of HIF-1α protein (Figure 5C and also in Figure 1A). No evidence of S-nitrosation was detected by analysis with the biotin switch technique, as shown in Figure 5C, in contrast to a previous report of direct modification of HIF-1α protein by exogenous NO.[13] On the other hand, S-nitrosated PHD2 was clearly detected in cells exposed to hypoxia for 4 h but not in cells grown in 21% O_2, and this observation was corroborated by demonstration of S-nitrosated PHD2 in cells exposed to exogenous NO for 3.6 h at a steady-state concentration of 1.74 μM NO, resulting in a cumulative dose of 400 μM·min (Figure 5D). These results show that modification of the enzyme PHD2 by RNS occurs during HIF-1α accumulation in vivo and, thus, may contribute to the enhanced stabilization of HIF-1α.

DISCUSSION

The aberrant activity of the transcription regulator HIF-1α has been associated with tumor progression,[1,2] and this pathway has been extensively investigated to identify avenues for development of improved therapeutic strategies. Among regulatory processes leading to HIF-1α stabilization, ROS and NO have been shown to exert multiple effects, depending on the experimental model studied.

The literature contains conflicting reports that NO either stabilizes or favors degradation of HIF-1α during hypoxia.[3,4,11] Studies based on exposure to an exogenous NO donor (DETA-NO) in hypoxia indicate that concentrations of NO below 400 nM decreased HIF-1α, while concentrations above 1 μM stabilized it.[4,11] The majority of previous studies in this context relied on exposure to relatively high concentrations of NO donor drugs (e.g., >50−100 μM DETA-NO) or on transfection-based expression of NOS.[4,8,11] Collectively, these approaches suggested that, under hypoxia, NO in the range of 400 nM decreased mitochondrial oxygen consumption, thereby raising intracellular oxygen availability for PHD activity, resulting in HIF-1α destabilization. In stably transfected iNOS-expressing cells submitted to hypoxia, the effect of NO on HIF-1α was shown to be biphasic, with decreased and increased HIF-1α stability at different NO concentrations.[7,11] This discrepancy in findings may be attributed not only to the distinct nature and regimen of NO donors employed but also to the complex chemistry of NO, which by interaction with ROS gives rise to numerous species such as $NO_2^{\bullet}$, peroxynitrite, N_2O_3, and *S*-nitrosothiols.[4,27]

Because autoxidation of 1 mol of NO yields 1 mol of NO_2^- (eq A1), it is tempting to suppose that the steady-state rate of NO_2^- formation equals the net rate of cellular NO synthesis (the rate of synthesis minus the rate of cellular consumption). However, this is far from true, especially in thin films of medium and at low concentrations of NO. Under these conditions, nearly all of the NO will diffuse out of the liquid before it can react and be trapped as NO_2^-. As derived in the Appendix, the fraction of NO lost from the liquid (f) is

$$f = 1 - \frac{2L^2}{D_{NO}}\left(\frac{kC_0R}{3}\right)^{1/2} \quad (2)$$

where D_{NO} is the aqueous diffusivity of NO. For our hypoxic conditions, f = 0.99 during both periods. That is, 99% of the NO leaving the cells was lost to the headspace. Moreover, although the rate of NO_2^- formation (R) indeed increases with the net rate of NO synthesis (N), the relationship between R and N is nonlinear. Without an appropriate reaction−diffusion model, only directional changes in the NO concentration or the rate of NO synthesis can be inferred from measured changes in R.

Increased ROS levels in cells have also been associated with HIF-1α stabilization,[28,29] and in our studies, exposure of HCT116 cells to 3% oxygen resulted in augmented generation of ROS along with increased accumulation of HIF-1α. Interestingly, concurrent treatment with an antioxidant had a limited effect on this response to hypoxia, pointing to the involvement of additional factors. Other studies of stabilization of HIF-1α by NO showed that PHD activity was inhibited as ROS production increased, and it was suggested that reciprocal scavenging by NO and ROS might therefore be a determinant of HIF-1α accumulation.[30]

Considering the poorly defined role of endogenous, low levels of NO generated in response to hypoxia, as well as the interplay between ROS and NO in the cellular redox environment, we sought to characterize the importance of NO in the processes leading to HIF-1α stabilization. In our experimental model, increased amounts of NO were detected in cells exposed to hypoxia for up to 6 h. We also found that iNOS protein expression and NO accumulation were significantly greater under hypoxia. Expression of eNOS and nNOS was inconsistent or showed no significant alteration.

We found that NOS inhibitors alone failed to inhibit HIF-1α stabilization completely, but treatments causing concomitant decreases in both ROS and NO production resulted in a marked increase in degradation of HIF-1α, in agreement with previous postulates that, in the hypoxic milieu, these species may act in concert to regulate HIF-1α.[23,24,27] In addition, also in agreement with a previous report, no variation in the expression of HIF-1α mRNA was seen upon treatment with the inhibitors above,[30] suggesting that ROS and NO regulate HIF-1α post-transcriptionally.

NO has the potential to nitrosate proteins at cysteine residues and, hence, to modulate their activity.[25] Metzen et al. showed that the thiol-reducing agent, DTT, decreased exogenous NO-induced HIF-1α stabilization caused by exogenously administered NO.[8] They postulated that *S*-nitrosothiols formed upon exposure to NO were critical for HIF-1α stabilization, even though intracellular targets for S-nitrosation were not demonstrated.[8] In our study, treatment with DTT, or conversely with DNCB, an agent that stabilizes cellular nitrosothiols, led to reduction and accumulation of HIF-1α protein respectively, suggesting that S-nitrosation was indeed involved in the process.

Although S-nitrosation of HIF-1α at Cys533 is known to prevent its breakdown,[13] we were unable to detect S-nitrosated HIF-1α in our model. Because HIF-1α hydroxylation was affected by ROS/NOS inhibitors (Figure 4A) and prompted by prior suggestions of PHDs as targets for regulation by S-nitrosation,[8,14] we investigated the occurrence of S-nitrosation of PHD2 in our model. Indeed, both hypoxia and exposure to controlled amounts of exogenous NO resulted in PHD2

nitrosation. Whether S-nitrosation alone, at levels detected in our experiments, reduces PHD2 activity to a degree sufficient to cause the observed effects on HIF stabilization, remains to be demonstrated by further investigation. Our results with the PHD inhibitor DMOG (Figure 4C) and increased HIF-1α hydroxylation in the presence of ROS/NOS inhibitors suggest that this may be the case. On the other hand, because NO is known to inhibit PHD by other mechanisms,[9] a combination of factors is likely to be involved.

The relevance of NO in stabilizing HIF-1α is evident in the context of cancer, since a variety of tumors have been shown to express NOS, and the accumulation of HIF-1α in those could be prevented by blocking NOS activity.[31−33] The additive effects of endogenously produced ROS and NO in the induction of post-translational modifications leading to HIF-1α stabilization demonstrated in our experiments have evident implications for development of therapeutics, inasmuch HIF-1α is a crucial component of cancer progression.

APPENDIX

It is desired to relate the NO concentration caused by NO biosynthesis to the rate of NO_2^- formation measured by assaying aliquots of cell culture medium. Competing with the diffusional loss of NO to the incubator gas is its multistep autoxidation, which is written overall as [21]

$$4NO + O_2 + 2H_2O \xrightarrow{k} 4NO_2^- + 4H^+ \quad \text{(A1)}$$

The rate constant k is such that the local rate of formation of NO_2^- per unit volume is $4k{C_{NO}}^2C_{O_2}$, where C_{NO} and C_{O_2} are the local NO and O_2 concentrations, respectively. Accordingly, for a liquid layer extending from $z = 0$ (dish surface) to $z = L$ (gas−liquid interface), the observable (average) rate of NO_2^- formation is

$$R = \frac{d\bar{C}_{NO_2^-}}{dt} = \frac{4k}{L}\int_0^L {C_{NO}}^2 C_{O_2}\, dz \quad \text{(A2)}$$

where $\bar{C}_{NO_2^-}$ is the NO_2^- concentration in a well-mixed sample. What is needed to evaluate the integral are $C_{NO}(z)$ and $C_{O_2}(z)$.

The scaled, dimensionless position and concentrations used hereafter are

$$\zeta = \frac{z}{L}, \quad \theta = \frac{C_{NO}}{C_1}, \quad \phi = \frac{C_{O_2}}{C_0} \quad \text{(A3)}$$

where C_0 is the aqueous O_2 concentration at the gas−liquid interface, as determined by the incubator gas and O_2 solubility. The reference concentration for NO is related to the net rate of NO synthesis per unit area of dish (N) and the aqueous NO diffusivity (D_{NO}) as $C_1 = NL/D_{NO}$.

With the new variables, the rate of NO_2^- formation is

$$R = 4k{C_1}^2C_0\int_0^1 \theta^2\phi\, d\zeta \quad \text{(A4)}$$

If the NO and O_2 concentrations were each uniform (at C_1 and C_0, respectively), the integral would equal unity and the NO_2^- formation rate would be just $R = 4k{C_1}^2C_0$.

The model used to predict the NO and O_2 concentration variations is a simplified version of that in Kim et al. in which there were liquid-filled chambers both above and below a porous membrane that supported the cells.[34] To describe the present experiments, it is necessary only to omit the relationships that applied to the lower chamber. Following the reasoning detailed in Kim et al., the effect of autoxidation on $C_{O_2}(z)$ is found to be negligible.[34] The O_2 concentration is then described by

$$\phi(\zeta) = 1 - A(1 - \zeta) \quad \text{(A5)}$$

The dimensionless parameter A equals the fractional drop in O_2 concentration within the liquid. It is related to the rate of respiratory consumption of O_2 per unit area of dish (M), the aqueous O_2 diffusivity (D_{O_2}), the film thickness, and the O_2 concentration as

$$A = \frac{ML}{C_0 D_{O_2}} \quad \text{(A6)}$$

The extremes of $A = 0$ and $A = 1$ correspond to relatively rapid and slow supply of O_2, respectively. The NO concentration is governed by

$$\frac{d^2\theta}{d\zeta^2} = B[1 - A(1 - \zeta)]\theta^2, \quad \frac{d\theta}{d\zeta}(0) = -1, \quad \theta(1) = 0 \quad \text{(A7)}$$

$$B = \frac{4kC_0C_1L^2}{D_{NO}} \quad \text{(A8)}$$

where the dimensionless parameter B is a measure of the rate of autoxidation of NO relative to diffusion. In the present experiments, as in Kim et al.,[34] a steady-state model is appropriate because the time required for diffusion of either species across the liquid film (about 5 min) is much shorter than the duration of the experiments (several hours).

A major additional simplification stems from the fact B in the present experiments is small. The parameter A was also found to be nearly zero. The small values of B and A are due to the thin films of medium, the moderate cell densities used, and the moderate rates of NO synthesis and O_2 consumption per cell. This suggests setting $A = B = 0$. In this case, the dimensionless NO and O_2 concentrations from eqs A7 and A5 are simply $\theta(\zeta) = 1 - \zeta$ and $\phi(\zeta) = 1$, respectively, and eq A4 reduces to

$$R = 4k{C_1}^2C_0\int_0^1 (1 - \zeta)^2\, d\zeta = \frac{4k{C_1}^2C_0}{3} \quad \text{(A9)}$$

Thus, the rate of NO_2^- formation is found to be one-third of what it would be for spatially uniform concentrations. The corresponding NO concentration at the cells is

$$C_{NO}(0) = C_1\theta(0) \quad \text{(A10)}$$

Because $\theta(0) = 1$ for $B = 0$, $C_{NO}(0) = C_1$ for this special case. Thus, solving eq A9 for C_1 gives eq 1.

As shown in Dendroulakis et al.,[35] very little NO reacts with the organics in cell culture media. Thus, almost all NO that escapes from the cells will either diffuse out of the liquid or undergo autoxidation. The fraction of NO that diffuses into the headspace is

$$f = 1 - \frac{RL}{N} \quad \text{(A11)}$$

Using eq A9 and the definition of C_1 to relate N to R leads to eq 2. Because R varies as N^2 (as a consequence of the autoxidation rate law), the fraction of NO lost from the system increases as N decreases. At low NO concentrations

(corresponding to small values of N), diffusional loss may be nearly complete.

Neither A nor B was precisely zero in our experiments. Using data on respiratory O_2 consumption for HCT116 cells in Chin et al.,[36] we estimate that $A = 0.064$ for the hypoxic cells. Calculating B in eq A8 by using eq A10 for C_1 gives $B = 0.035$. A numerical solution of eq A7 using these values indicates that eq 1 is accurate to within 1% for our experiments. The parameter values used were $k = 2.4 \times 10^6\ M^{-2}\ s^{-1}$ from Lewis and Deen,[21] C_0(control) = 223 μM from Chin et al.,[36] C_0(hypoxia) = 32 μM, $D_{NO} = 3.0 \times 10^{-5}\ cm^2/s$ from Zacharia and Deen,[37] and $D_{O_2} = 2.8 \times 10^{-5}\ cm^2/s$ from Goldstick and Fatt.[38] A volume of 3 mL was placed in dishes of 60 mm diameter, corresponding to $L = 1.06$ mm. For additional information on the evaluation of A, including respiratory inhibition by NO, see Kim et al.[34]

AUTHOR INFORMATION

Corresponding Author

*Tel: 617-253-3188. Fax: 617-324-5698. E-mail: wogan@mit.edu.

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ABBREVIATIONS

HIF-1α, hypoxia-inducible factor-1α; PHDs, prolyl hydroxylases; NO, nitric oxide; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; L-NMMA, N^G-monomethyl L-arginine; VHL, von Hippel-Lindau protein; NNA, nitro-L-arginine; PTIO, 2-4-carboxyphenyl-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide; DMOG, dimethyloxaloylglycine; DTT, dithiothreitol; 1400W, *N*-[(3-aminomethyl)benzyl]-acetamidine; DNCB, dichloronitrobenzene; CM-H2DCFDA, 5-(or-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetateacetyl ester; iNOS, inducible nitric oxide synthase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

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