Published in final edited form as:

Chem Res Toxicol. 2012 October 15; 25(10): 2194–2202. doi:10.1021/tx300274a.

Nitric oxide produced endogenously is responsible for hypoxia-induced HIF-1 α stabilization in colon carcinoma cells

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

Rajdeep Chowdhury: rajdeep2@mit.edu; Luiz C. Godoy: lcgodoy@mit.edu; Apinya Thiantanawat: apinya@cri.or.th; Laura J. Trudel: ljtrudel@mit.edu; William M. Deen: wmdeen@mit.edu; Gerald N. Wogan: wogan@mit.edu

[†]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139 [§]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139 [‡]Chulabhorn Research Institute, Laboratory of Pharmacology, Bangkok 10210 Thailand

Abstract

Hypoxia-inducible factor-1 alpha (HIF-1 α) is a critical regulator of cellular responses to hypoxia. Under normoxic conditions, cellular HIF-1 α 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-1 α , and thus NO is suggested to mimic hypoxia. However, the role of low levels of endogenously produced NO generated during hypoxia in HIF-1 α 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-1 α accumulation in hypoxia. The antioxidant N-acetyl-L-cysteine (NAC) and NO synthase inhibitor N^G-monomethyl L-arginine (L-NMMA) effectively reduced HIF-1 α stabilization and decreased HIF-1 α 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-1 α stabilization in hypoxic cells, while dinitrochlorobenzene, which stabilizes S-nitrosothiols, favored its accumulation. This suggested that ROS- and NO-mediated HIF-1 α stabilization involved S-nitrosation, which was confirmed by demonstrating increased S-nitrosation of PHD2 during hypoxia. Our results support a regulatory mechanism of HIF-1 α during hypoxia in which endogenously generated NO and ROS promote inhibition of PHD2 activity, probably by its S-nitrosation.

Keywords

hypoxia; nitric oxide; reactive oxygen species; HIF-1 α ; PHD-2; S-nitrosation

INTRODUCTION

HIF-1 α is a transcription factor that plays a key role in regulating cellular functions in response to variations in tissue oxygen tension. HIF-1 α 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), ubiquitination and proteosomal degradation. When hypoxia develops, as in poorly vascularized solid tumors, HIF-1 α levels are augmented, causing increased expression of HIF-1 α -regulated genes that contribute to enhanced tumor cell growth, angiogenesis and resistance to chemotherapeutic agents.^{1, 2}

Based on 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-1 α , 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 O₂ 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, non-uniform 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-1 α 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-1 α interaction with VHL and hence HIF-1 α stability.^{4, 5, 9, 10} In relating various cellular responses to NO exposures, Thomas et al³ 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 to NONOates or to co-culture 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-1 α 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-1 α stabilization during hypoxia, and found among other effects S-nitrosation of PHD2, which was in turn associated with lower HIF-1 α hydroxylation and 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, N C I, Bethesda, MD) were cultured at 37°C, 5% CO₂ 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 control.

For hypoxia treatment, 3×10^6 cells in 3 ml fresh medium were placed in an air-tight Modular Incubator Chamber (Billups-Rothenberg, Inc.), flushed with gas comprising 3% O₂, 5% CO₂ and 92% N₂ 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% O₂, 5% CO₂, and 74% N₂. Cell-culture reagents were purchased from Lonza and gases 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-1H-imidazolyl-1-oxy-3-oxide (PTIO, 300 µM); anti-oxidant, N-acetyl-L-cysteine (NAC, 5 mM); proteasome inhibitor, MG132 (10 µM); competitive PHD inhibitor, dimethylloxaloylglycine (DMOG, 1 mM) and thiol-reductant, dithiothreitol (DTT, 0.5 – 1.5 mM). In some specific experiments, cells were exposed to selective 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 performed in our lab.^{17, 18}

Detection of ROS and NO

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 washing twice with PBS, fluorescence intensity was measured in an automatic plate reader (Spectra Max Gemini, Molecular Device).

For detection of NO by microscopy, after treatments cells were incubated with the NO-specific fluorescent dye diaminofluorescein-2 diacetate (DAF-2DA) following 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 NO₂⁻ 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. Nitric oxide 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 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 re-hydrated with PBS. Nonspecific binding sites were blocked with 5% non-fat dry milk for 1 h, followed by overnight incubation with primary antibody (anti-HIF-1 α ; 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, then transfected using Lipofectamine Reagent (Invitrogen) with plasmid DNA (0.5 μg). Inducible HIF-1 α -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 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 pmoles of siRNA duplex corresponding to HIF-1 α following 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 protein content 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: anti-hydroxy-HIF-1 α (Pro564) (Cell Signaling Technology); anti-Histone H1 (Santa Cruz Biotechnology); anti-PHD2 (Abcam); and anti-HIF-1 α (BD Transduction Laboratories). Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG or rabbit anti-goat IgG (Santa Cruz Biotechnology). As a protein loading control, membranes were stripped and re-probed with anti-mouse β -actin monoclonal antibody (Sigma). HIF-1 α detection by western blot was done by processing lysates in less than 3 minutes 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 h or 4 h) or exogenous NO (1.8 h 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% CHAPS, 0.1% SDS, and protease inhibitors. Lysates were centrifuged at $14,000 \times g$, and 1 mg total protein from each sample was precipitated with cold acetone and re-suspended 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 thiolreductant 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, $C_{\text{NO}}(z)$. 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

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

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 three to four 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-1 α accumulation

Upon oxygen deprivation of cells, HIF-1 α 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% O₂) or 21% O₂ 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 Figs. 1A and 1B, exposure for 2 to 6 h induced a gradual increase in HIF-1 α protein level, as well as its translocation into the nucleus.

Generation of ROS and NO during hypoxia

It has been proposed that ROS generation is a prerequisite for HIF-1 α stabilization under hypoxia.^{5, 10} We detected a significant increase in intracellular ROS content upon exposure of cells to hypoxia for 4 to 6 h (Fig. 2A). Mitochondria were identified as the source of ROS (data not shown), and ROS levels were significantly attenuated by the antioxidant NAC (Fig. 2A). Endogenous cellular NO concentrations were calculated from the rates of NO₂⁻ accumulation using Eq. (1). For the controls (21% O₂), there was not a significant increase in NO₂⁻ concentration over time (Fig. 2B). Accordingly, the NO concentration was undetectable. The progressively increasing NO₂⁻ concentrations for hypoxic cells (3% O₂, Fig. 2B) gave average NO₂⁻ 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 μ M (Fig. 2B). Thus, for the hypoxic cells NO concentration remained at about 0.3 μ M 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 during exposure of cells to hypoxia for 6 h. Moreover, existence of a positive HIF-1 α -iNOS feedback loop is evident from the data, which shows that silencing of HIF-1 α by si-RNA led to concomitant reduction in iNOS protein levels suggesting that iNOS may be under transcriptional control of HIF-1 α .

Endogenous ROS and NO sustain HIF-1 α accumulation

HIF-1 α content was analyzed in hypoxic cells pre-treated with NOS inhibitors (L-NMMA and NNA), an NO scavenger (PTIO), an antioxidant (NAC) or a combination of L-NMMA and NAC (Fig. 3A). While all treatments resulted in diminished stabilization of HIF-1 α , the combination of L-NMMA and NAC was most effective in preventing its accumulation in whole cell extract as well as in the nucleus (Fig. 3A). When treated with the iNOS specific inhibitor 1400W cells showed similar results, with enhanced degradation of HIF-1 α in the presence of 1400W and NAC compared to iNOS inhibitor alone (Fig. 3A). Furthermore, cells transfected with a HIF-1 α -dependent luciferase reporter construct showed marked reduction in luciferase activity following L-NMMA and NAC treatment and hypoxia, indicating decreased HIF-1 α transcriptional activity (Fig. 3B). Semi-quantitative RT-PCR showed no change in transcription of the HIF-1 α 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 PHD activity is decreased, resulting in HIF-1 α accumulation.^{1, 2} To assess contributions of NO and ROS to this process, we analyzed levels of hydroxylated HIF-1 α 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-1 α . As shown in Fig. 4A, higher levels of hydroxylated HIF-1 α were found in hypoxic cells treated with L-NMMA and NAC than in untreated hypoxic cells. These results suggest that NO and ROS lead to increased HIF-1 α stabilization in hypoxia by inhibiting HIF-1 α 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-1 α hydroxylation. PHDs have several essential cofactors, such as 2-oxoglutarate, and the oxoglutarate analog dimethyloxaloylglycine (DMOG) constitutes a potent inhibitor of PHD activity.²² As shown in Fig. 3A, L-NMMA and NAC treatment reduced HIF-1 α stabilization during hypoxia. On the other hand, when PHD was inhibited by DMOG, L-NMMA and NAC failed to reduce HIF-1 α stabilization (Fig. 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-1 α (Fig. 5A). On the other hand, when cells were treated with DNFB, which favors stabilization of cellular S-nitrosothiols, increased HIF-1 α accumulation was seen when compared to cells exposed to hypoxia alone (Fig. 5B). These results led us to hypothesize that thiol-sensitive mechanisms such as S-nitrosation are involved in HIF-1 α stabilization.

Accordingly, we analyzed S-nitrosation levels in the HIF-1 α 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-1 α protein (Fig. 5C and also in Fig. 1A). No evidence of S-nitrosation was detected by analysis with the biotin switch technique, as shown in Fig. 5C, in contrast to a previous report of direct modification of HIF-1 α protein by exogenous NO¹³. 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% O₂, 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 μ M NO, resulting in a cumulative dose of 400 μ M.min (Fig. 5D). These results show that modification of the enzyme PHD2 by RNS occurs during HIF-1 α accumulation *in vivo*, and thus may contribute to the enhanced stabilization of HIF-1 α .

DISCUSSION

Aberrant activity of the transcription regulator HIF-1 α 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-1 α 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-1 α during hypoxia.^{3, 4, 11} Studies based on exposure to an exogenous NO donor (DETA-NO) in hypoxia indicates that concentrations of NO below 400 nM decreased HIF-1 α , while concentrations above 1 μ M 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 μ M 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-1 α destabilization. In stably transfected iNOS-expressing cells submitted to hypoxia, the effect of NO on HIF-1 α was shown to be biphasic, with decreased and increased HIF-1 α 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 NO₂[•], peroxynitrite, N₂O₃ and S-nitrosothiols.^{4, 27}

Because autoxidation of one mole of NO yields one mole of NO₂[−] [Eq. (A1)], it is tempting to suppose that the steady-state rate of NO₂[−] 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 NO₂[−]. As derived in the Appendix, the fraction of NO lost from the liquid (f) is

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

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 NO₂[−] 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-1 α 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-1 α . 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-1 α 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-1 α 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-1 α stabilization. In our experimental model, increased amounts of NO were detected in cells exposed to hypoxia for up to 6 hours. 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-1 α stabilization completely, but treatments causing concomitant decreases in both ROS and NO production resulted in a marked increase in degradation of HIF-1 α , in agreement with previous postulates that, in the hypoxic milieu, these species may act in concert to regulate HIF-1 α .^{23, 24, 27} In addition, also in agreement with a previous report, no variation in the expression of HIF-1 α mRNA

was seen upon treatment with the inhibitors above,³⁰ suggesting that ROS and NO regulate HIF-1 α 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-1 α stabilization caused by exogenously administered NO.⁸ They postulated that S-nitrosothiols formed upon exposure to NO were critical for HIF-1 α 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-1 α protein respectively, suggesting that S-nitrosation was indeed involved in the process.

Although S-nitrosation of HIF-1 α at Cys533 is known to prevent its breakdown,¹³ we were unable to detect S-nitrosated HIF-1 α in our model. Because HIF-1 α hydroxylation was affected by ROS/NOS inhibitors (Fig. 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 (Fig. 4C) and increased HIF-1 α hydroxylation in presence of ROS/NOS inhibitors suggest that this may be the case. On the other hand, since 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-1 α is evident in the context of cancer, since a variety of tumors have been shown to express NOS, and the accumulation of HIF-1 α 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-1 α stabilization demonstrated in our experiments have evident implications for development of therapeutics, inasmuch HIF-1 α is a crucial component of cancer progression.

Acknowledgments

This work was supported by National Cancer Institute Program Project Grant 5 P01 CA26731 and National Institute of Environmental Health Sciences Center Grant ES02109.

Appendix

It is desired to relate the NO concentration caused by NO biosynthesis to the rate of NO₂[−] 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 as²¹

The rate constant k is such that the local rate of formation of NO₂[−] per unit volume is $4kC_{\text{NO}}^2C_{\text{O}_2}$, where C_{NO} and C_{O_2} are the local NO and O₂ concentrations, respectively. Accordingly, for a liquid layer extending from $z = 0$ (dish surface) to $z = L$ (gas-liquid interface), the observable (average) rate of NO₂[−] formation is

$$R = \frac{d\bar{C}_{\text{NO}_2^-}}{dt} = \frac{4k}{L} \int_0^L C_{\text{NO}}^2 C_{\text{O}_2} dz \quad (\text{A2})$$

where $\bar{C}_{\text{NO}_2^-}$ is the NO_2^- concentration in a well-mixed sample. What is needed to evaluate the integral are $C_{\text{NO}}(z)$ and $C_{\text{O}_2}(z)$.

The scaled, dimensionless position and concentrations used hereafter are

$$\zeta = \frac{z}{L}, \theta = \frac{C_{\text{NO}}}{C_1}, \phi = \frac{C_{\text{O}_2}}{C_0} \quad (\text{A3})$$

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 $C_1 = NL/D_{\text{NO}}$. With the new variables, the rate of NO_2^- formation is

$$R = 4kC_1^2C_0 \int_0^1 \theta^2 \phi d\zeta. \quad (\text{A4})$$

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 $R = 4kC_1^2C_0$.

The model used to predict the NO and O_2 concentration variations is a simplified version of that in Kim et al. (2012) 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. (2012), the effect of autoxidation on $C_{\text{O}_2}(z)$ 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

$$A = \frac{ML}{C_0 D_{\text{O}_2}}. \quad (\text{A6})$$

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

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

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

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. (2012),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 O₂ consumption per cell. This suggests setting $A = B = 0$. In this case the dimensionless NO and O₂ concentrations from Eqs. (A7) and (A5) are simply $\theta(\zeta) = 1 - \zeta$ and $\phi(\zeta) = 1$, respectively, and Eq. (A4) reduces to

$$R = 4kC_1^2C_0 \int_0^1 (1 - \zeta)^2 d\zeta = \frac{4kC_1^2C_0}{3}. \quad (\text{A9})$$

Thus, the rate of NO₂⁻ formation is found to be one third of what it would be for spatially uniform concentrations. The corresponding NO concentration at the cells is

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

Because $\theta(0) = 1$ for $B = 0$, $C_{\text{NO}}(0) = C_1$ 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 O₂ consumption for HCT116 cells in Chin et al. (2010),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 $k = 2.4 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$ from Lewis and Deen,²¹ $C_0(\text{control}) = 223 \text{ } \mu\text{M}$ from Chin et al.,³⁶ $C_0(\text{hypoxia}) = 32 \text{ } \mu\text{M}$, $D_{\text{NO}} = 3.0 \times 10^{-5} \text{ cm}^2/\text{s}$ from Zacharia and Deen,³⁷ and $D_{\text{O}_2} = 2.8 \times 10^{-5} \text{ cm}^2/\text{s}$ from Goldstick and Fatt.³⁸ A volume of 3 mL was placed in dishes of 60 mm diameter, corresponding to $L = 1.06 \text{ mm}$. For additional information on the evaluation of A , including respiratory inhibition by NO, see Kim et al. (2012).³⁴

List of Abbreviations

HIF-1α	hypoxia-inducible factor-1 alpha
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-1H-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 diacetate, acetyl ester
iNOS	inducible nitric oxide synthase
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

References

- Quintero M, Mackenzie N, Brennan PA. Hypoxia-inducible factor 1 (HIF-1) in cancer. *Eur. J Surg. Oncol.* 2004; 30:465–468. [PubMed: 15135470]
- Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl. Physiol.* 2000; 88:1474–1480. [PubMed: 10749844]
- Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolucci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA. The chemical biology of nitric oxide: implications in cellular signaling. *Free Radical Biol. & Med.* 2008; 45:18–31. [PubMed: 18439435]
- Brune B, Zhou J. Nitric oxide and superoxide: interference with hypoxic signaling. *Cardiovascular Res.* 2007; 75:275–282.
- Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1 α during hypoxia: a mechanism of O₂ sensing. *J Biol. Chem.* 2000; 275:25130–25138. [PubMed: 10833514]
- Kaelin WG Jr. ROS: really involved in oxygen sensing. *Cell Metab.* 2005; 1:357–358. [PubMed: 16054083]
- Mateo J, Garcia-Lecea M, Cadenas S, Hernandez C, Moncada S. Regulation of hypoxia-inducible factor-1 α by nitric oxide through mitochondria-dependent and -independent pathways. *Biochem. J.* 2003; 376:537–544. [PubMed: 14531732]
- Metzen E, Zhou J, Jelkmann W, Fandrey J, Brune B. Nitric oxide impairs normoxic degradation of HIF-1 α by inhibition of prolyl hydroxylases. *Molec. Biol. Cell.* 2003; 14:3470–3481. [PubMed: 12925778]
- Kaelin WG Jr, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Molecular Cell.* 2008; 30:393–402. [PubMed: 18498744]
- Schroedl C, McClintock DS, Budinger GR, Chandel NS. Hypoxic but not anoxic stabilization of HIF-1 α requires mitochondrial reactive oxygen species. *Amer. J Physiol.* 2002; 283:L922–L931.
- Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1 α . *Science (New York, N.Y.)* 2003; 302:1975–1978.

12. Hampl V, Cornfield DN, Cowan NJ, Archer SL. Hypoxia potentiates nitric oxide synthesis and transiently increases cytosolic calcium levels in pulmonary artery endothelial cells. *Eur. Respir. J.* 1995; 8:515–522. [PubMed: 7664847]
13. Li F, Sonveaux P, Rabbani ZN, Liu S, Yan B, Huang Q, Vujaskovic Z, Dewhirst MW, Li CY. Regulation of HIF-1 α stability through S-nitrosylation. *Molecular Cell.* 2007; 26:63–74. [PubMed: 17434127]
14. Chowdhury R, Flashman E, Mecinovic J, Kramer HB, Kessler BM, Frapart YM, Boucher JL, Clifton IJ, McDonough MA, Schofield CJ. Studies on the reaction of nitric oxide with the hypoxia-inducible factor prolyl hydroxylase domain 2 (EGLN1). *J.Molecul. Biol.* 2011; 410:268–279.
15. Li CQ, Kim MY, Godoy LC, Thiantanawat A, Trudel LJ, Wogan GN. Nitric oxide activation of Keap1/Nrf2 signaling in human colon carcinoma cells. *Proc. Natl. Acad. Sci.,USA.* 2009; 106:14547–14551. [PubMed: 19706542]
16. Wang C, Deen WM. Nitric oxide delivery system for cell culture studies. *Ann. Biomed. Eng.* 2003; 31:65–79. [PubMed: 12572657]
17. Li CQ, Trudel LJ, Wogan GN. Nitric oxide-induced genotoxicity, mitochondrial damage, and apoptosis in human lymphoblastoid cells expressing wild-type and mutant p53. *Proc. Natl. Acad. Sci.,USA.* 2002; 99:10364–10369. [PubMed: 12136132]
18. Li CQ, Trudel LJ, Wogan GN. Genotoxicity, mitochondrial damage, and apoptosis in human lymphoblastoid cells exposed to peroxynitrite generated from SIN-1. *Chem. Res. Toxicol.* 2002; 15:527–535. [PubMed: 11952339]
19. Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, Jourdain D, Kelm M. Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *FASEB.* 2002; 16:1775–1785.
20. Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE.* 2001; 2001:pl1. [PubMed: 11752655]
21. Lewis RS, Deen WM. Kinetics of the reaction of nitric oxide with oxygen in aqueous solutions. *Chem. Res. Toxicol.* 1994; 7:568–574. [PubMed: 7981422]
22. Asikainen TM, White CW. HIF stabilizing agents: shotgun or scalpel? *American journal of physiology. Lung Cellul. Molecul.Physiol.* 2007; 293:L555–L556.
23. Darley-Usmar V, Wiseman H, Halliwell B. Nitric oxide and oxygen radicals: a question of balance. *FEBS letters.* 1995; 369:131–135. [PubMed: 7649244]
24. Halliwell JV, Grove EA. 9-Amino-1,2,3,4-tetrahydroacridine (THA) blocks agonist-induced potassium conductance in rat hippocampal neurones. *Eur. J. Pharma.* 1989; 163:369–372.
25. Mannick JB, Schonhoff C, Papeta N, Ghafourifar P, Szibor M, Fang K, Gaston B. S-Nitrosylation of mitochondrial caspases. *J. Cell Biol.* 2001; 154:1111–1116. [PubMed: 11551979]
26. Chakraborti D, Sengupta MK, Rahman MM, Ahamed S, Chowdhury UK, Hossain MA, Mukherjee SC, Pati S, Saha KC, Dutta RN, Quamruzzaman Q. Groundwater arsenic contamination and its health effects in the Ganga-Meghna-Brahmaputra plain. *J. Environ. Monit.* 2004; 6:74N–83N.
27. Murphy MP. Does interplay between nitric oxide and mitochondria affect hypoxia-inducible transcription factor-1 activity? *Biochem. J.* 2003; 376:e5–e6. [PubMed: 14627433]
28. Fong GH, Takeda K. Role and regulation of prolyl hydroxylase domain proteins. *Cell Death Dif.* 2008; 15:635–641.
29. Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ, Simon MC. Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. *Molecul. Cell. Biol.* 2007; 27:912–925.
30. Kohl R, Zhou J, Brune B. Reactive oxygen species attenuate nitric-oxide-mediated hypoxia-inducible factor-1 α stabilization. *Free Radical Biol.& Med.* 2006; 40:1430–1442. [PubMed: 16631533]
31. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. *Natl. Rev. Cancer.* 2006; 6:521–534.
32. Quintero M, Brennan PA, Thomas GJ, Moncada S. Nitric oxide is a factor in the stabilization of hypoxia-inducible factor-1 α in cancer: role of free radical formation. *Cancer Res.* 2006; 66:770–774. [PubMed: 16424008]

33. Weigert A, Brune B. Nitric oxide, apoptosis and macrophage polarization during tumor progression. *Nitric Oxide*. 2008; 19:95–102. [PubMed: 18486631]
34. Kim MY, Lim CH, Trudel LJ, Deen WM, Wogan GN. Delivery method, target gene structure, and growth properties of target cells impact mutagenic responses to reactive nitrogen and oxygen species. *Chem. Res. Toxicol.* 2012; 25:873–883. [PubMed: 22303861]
35. Dendroulakis V, Russell BS, Eric Elmquist C, Trudel LJ, Wogan GN, Deen WM, Dedon PC. A system for exposing molecules and cells to biologically relevant and accurately controlled steady-state concentrations of nitric oxide and oxygen. *Nitric Oxide*. 2012; 27:161–168. [PubMed: 22728703]
36. Chin MP, Schauer DB, Deen WM. Nitric oxide, oxygen, and superoxide formation and consumption in macrophages and colonic epithelial cells. *Chem. Res. Toxicol.* 2010; 23:778–787. [PubMed: 20201482]
37. Zacharia IG, Deen WM. Diffusivity and solubility of nitric oxide in water and saline. *Ann. Biomed. Eng.* 2005; 33:214–222. [PubMed: 15771275]
38. Goldstick TK, Fatt I. Diffusion of Oxygen in Solutions of Blood Proteins. *Chem. Eng. Progr.* 1970; 66:101–107.

A **B**

Fig. 1. Hypoxia induces HIF-1α accumulation in HCT116 cells. (A) HIF-1α protein accumulation following exposure of cells to 3% O₂. (B) Nuclear localization of HIF-1α visualized through immunofluorescent staining; arrows indicate HIF-1α localized in the nucleus of cells exposed to 3% O₂ for 6 h.

Fig. 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% O₂, 3% O₂ or 3% O₂ 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: $P < 0.00004$; 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 4h and 6h over time compared to 2h at 3% O₂.

(C) Changes in levels of iNOS and HIF-1 α protein (western blot) caused by exposure of cells to 3% O₂ (6 h) and knockdown with siRNA specific for HIF-1 α .

Fig. 3. ROS and NO induce hypoxic HIF-1α accumulation and increased transcriptional activity. (A) Cells were exposed to 3% O₂ or 3% O₂ 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% O₂, 6 h-induced expression of HIF-1α-dependent promoter luciferase construct. The symbol * indicates significantly different values in inhibitor treated samples compared to 3% O₂, P < 0.01.

Fig. 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% O₂ 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 presence/absence of L-NMMA and NAC during 6 h exposure to 3% O₂. Protein extracts were analyzed for HIF-1α by western blot.

Fig. 5.

Evidence for S-nitrosation of PHD2. (A) Hypoxia-induced HIF-1α accumulation was blocked by treatment with 0 to 1.5 mM DTT to destabilize nitrosothiols during exposure of HCT116 cells to 3% O₂ for 4 h. (B) Hypoxia-induced HIF-1α accumulation was restored by treatment with DNCB, which stabilizes cellular nitrosothiols, during exposure to 3% O₂ for 1 or 2 h. (C) HIF-1α protein was stabilized by exposure to 3% O₂ 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% O₂. 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.