Nitric Oxide Modulates Oxygen Sensing by Hypoxia-inducible Factor 1-dependent Induction of Prolyl Hydroxylase 2*

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The transcription factor complex hypoxia-inducible factor 1 (HIF-1) plays a crucial role in cellular adaptation to low oxygen availability. O2-dependent HIF prolyl hydroxylases (PHDs) modify HIF-1α, which is sent to proteasomal degradation under normoxia. Reduced activity of PHDs under hypoxia allows stabilization of HIF-1α and induction of HIF-1 target gene expression. Like hypoxia, nitric oxide (NO) was found to inhibit normoxic PHD activity leading to HIF-1α accumulation. In contrast under hypoxia, NO reduced HIF-1α levels due to enhanced PHD activity. Herein, we studied the role of NO in regulating PHD expression and the consequences thereof for HIF-1α degradation. We report a biphasic response of HIF-1α and PHDs to NO treatment both under normoxia and hypoxia. In the early phase, NO inhibits PHD activity that leads to HIF-1α accumulation, whereas in the late phase, increased PHD levels reduce HIF-1α. NO induces expression of PHD2 and -3 mRNA and protein under normoxia and hypoxia in a strictly HIF-1-dependent manner. NO-treated cells with elevated PHD levels displayed delayed HIF-1α accumulation and accelerated degradation of HIF-1α upon reoxygenation. Subsequent suppression of PHD2 and -3 expression using small interfering RNA revealed that PHD2 was exclusively responsible for regulating HIF-1α degradation under NO treatment. In conclusion, we identified the induction of PHD2 as an underlying mechanism of NO-induced degradation of HIF-1α.

Shortage of oxygen, hypoxia, requires the initiation of adaptive mechanisms of cells to either improve transport of oxygen to tissues or to ensure sufficient ATP generation through glycolytic pathways (1). Central for the adaptation to hypoxia is the transcription factor hypoxia-inducible factor-1 (HIF-1), which up-regulates the expression of genes in control of angiogenesis, erythropoiesis, and glycolysis. HIF-1 is composed of an O2-regulated α-subunit (HIF-1α) and a constitutive β-subunit (HIF-1β). Two other orthologs of HIF-1α, termed HIF-2α and HIF-3α, as well as HIF-1β (identical to the previously described aryl hydrocarbon nuclear translocator Arnt) are members of the large family of basic helix-loop-helix-Per-Arnt-Sim transcription factors (2, 3). Under normoxic conditions, a family of prolyl hydroxylase domain-containing enzymes (PHD1, PHD2, PHD3, and potentially PH4) hydroxylate two proline residues (Pro-402 and Pro-564) in the oxygen-dependent degradation domain of HIF-1α (4–6), upon which the protein is recognized by the von Hippel-Lindau protein E3 ubiquitin ligase complex and targeted to proteasomal degradation (4, 5, 7–9). In addition to regulating HIF-1α protein stability, transcriptional activity of HIF-1α is regulated by hydroxylation of the Asn-803 residue within the C-terminal trans-activating domain of HIF-1α by the asparagyl hydroxylase named factor inhibiting HIF (FIH-1) (10), which impedes binding of the coactivator p300/CREB-binding protein (11). By hydroxylating HIF-1α in an oxygen-dependent manner, PHDs and FIH-1 function as oxygen sensors (12–14). So far, it has been reported that two of the known HIF hydroxylases, PHD2 and PHD3, themselves are hypoxia-inducible (12, 15), which is mainly due to the action of HIF-1α (16, 17). Thus, HIF-1-dependent PHD induction forms an autoregulatory loop controlling HIF-1α stability under prolonged hypoxia and upon reoxygenation (18–22).

Besides hypoxia, nitric oxide (NO) and/or NO-derived species regulate HIF-1α abundance and activity. Recent studies indicate that under normoxic conditions, chemically diverse NO donors, enhanced NO formation from inducible NO-synthase, or NO formation in a coculture system induced HIF-1α stabilization and transcriptional activation of HIF-1 target gene expression (23–25). Both increased protein synthesis due to NO-induced phosphatidylinositol 3-kinase or mitogen-activated protein kinase signaling (26, 27) and decreased degradation of HIF-1α by NO-dependent inhibition of PHD1, -2, and -3 activity (28) have been reported to contribute to this effect.

However, under hypoxic conditions, NO appears to have an opposite role in regulating HIF-1α, because several NO donors were found to decrease HIF-1α stabilization and HIF-1 transcriptional activation (29–31). It was reported that mitochondria play a role in NO regulation of HIF expression under hypoxia (32–35). Hagen et al. (33) proposed that mitochondrial oxygen consumption was reduced by NO inhibition of the cytochrome c oxidase. This would leave more oxygen for PHDs to regain activity and increase the degradation of HIF-1α. Alternatively, the inhibitory effect of NO on hypoxia-induced HIF-1α was explained by increased NO-derived species and/or reactive oxygen species that contribute to destabilization of HIF-1α by reactivation of PHD activity (36–38). In addition, calcium and calpain were found to contribute to the degradation of HIF-1α by NO under hypoxia (39). Interestingly, NO...
increased the activity of PHDs that were inhibited by hypoxia-mimicking agents like CoCl₂ (40) or desferrioxamine (37).

NO may thus affect PHD function at various steps depending on the cellular microenvironment. Since it was found that abundance and distribution of the PHD isoforms contribute to the regulation of HIF-1α in vivo (13, 19, 41), we studied the role of NO in regulating PHD expression and activity of HIF-1α. Herein we report a biphasic effect of NO on HIF-1α by (i) early induction through inhibition of PHD activity and (ii) later reduction by increased PHD2 protein amounts. Independently from other proposed mechanisms, we identified the induction of PHD2 as an underlying mechanism of NO-induced degradation of HIF-1α.

**EXPERIMENTAL PROCEDURES**

Reagents—S-Nitrosoglutathione (GSNO) was synthesized as described previously (24).

Cell Culture—The human osteosarcoma cells (U2OS) were a kind gift from J. M. Gleadle and P. Ratcliffe (Oxford, UK). U2OS cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin in a normoxic atmosphere of 21% O₂, 74% N₂, and 5% CO₂ (by volume). For cell culture experiments, cells were either exposed to normoxia or placed in a hypoxic incubator with 1% O₂, 94% N₂, 5% CO₂ (Heraeus incubator, Hanau, Germany) for variable periods of time. In case of experiments where GSNO or MG132 were added under hypoxic conditions (1% O₂, 94% N₂, 5% CO₂) a hypoxic work station (Invivo2 400, Ruskinn, Leicester, UK) was used.

Western Blot Analysis—Whole cell protein extracts were prepared from 35-mm dishes of cells that were about 80% confluent. Cells were lysed in 50 μl of extraction buffer (300 mM sodium chloride, 10 mM Tris (pH 7.9), 1 mM EDTA, 0.1% Nonidet P-40, 1% protease inhibitor mixture) (Roche Applied Science) for 20 min on ice and centrifuged (3600 × g at 4°C for 5 min). The supernatant was used as whole cell extract. The protein concentration was determined with a commercial protein method from 6-well dishes as described previously (43).

Reverse Transcription and Quantitative Real Time PCR—Total RNA was extracted using the guanidinium isothiocyanate method from 6-well dishes as described previously (43). 1 μg total RNA was reverse transcribed with oligo(dT) and Moloney murine leukemia virus reverse transcriptase (Promega). Human PHD1, -2, and -3 cDNAs and the cDNA of the housekeeping gene 60 S acidic ribosomal protein was quantified by real time PCR using the qPCR™ master mix for SYBR Green I (Eurorgenetics, Verviers, Belgium) and the GeneAmp5700 sequence Detection System (PerkinElmer Life Sciences). The PCRs were set up in a final volume of 25 μl with 0.5 μl of cDNA in 1× reaction buffer with SYBR Green I, 10 pmol of forward primer, and 10 pmol of reverse primer. The primers used were as follows: human PHD2, forward (5'-GCACGACACCGGGAAGTT-3') and reverse (5'-CAGCTTCCCGTTACAGT-3'); human PHD3, forward (5'-GGCCATCAGCTTCCCTGCTG-3') and reverse (5'-GGTGTGTAACACGCCAACTCCGTACAGT-3'); human PHD1, forward (5'-GGCGATCCCGCCGCG-3') and reverse (5'-AGAGGTTGCAAGGAGGAC-3') and human 60 S acidic ribosomal protein, forward (5'-ACGA-GGTGTGCAAGGAGGAC-3') and reverse (5'-GCAAGTCTGC-TTCCCATCTGC-3'). The PCR amplification profile was as follows: 10 min at 95°C followed by 30 cycles 15 s at 95°C and 1 min at 60°C. Agarose gel electrophoresis, purification, and DNA sequencing confirmed the identity of the PCR products. 10-Fold dilutions of purified PCR products starting at 1 pg to 0.1 fg were used as standards. The quantified cDNA of the PHDs were normalized to cDNA of the 60 S acidic ribosomal protein and expressed as normalized PHD mRNA level. All reverse transcription-PCRs were done in triplicate from RNA from three separate culture dishes.

Short Interfering RNA (siRNA) Treatment—For siRNA experiments, cells were 30–50% confluent and transfected with siRNAs using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. siRNA sequence targeting HIF-1α has been described previously (44). Sequences designed to suppress expression of PHD2 (sense, 5'-CAAGGUAAGGAGGAUGUAUU-3'; antisense, 5'-UAUACCUCACUUACCUUGUUGU-3'; GenBank™ accession number, EGLN1 NM_022051), PHD3 (sense, 5'-GUAAUUUGAUGAGGAAGUAAUU-3'; antisense, 5'-UUCUUCAGCAUCAGAAUGAAUU-3'; GenBank™ accession number, EGLN3 NM_022073), and luciferase (siControl nontargeting siRNA) were purchased from Dharmacon. Cells were transfected once with siRNA directed against HIF-1α (100 nM) at 24 h or twice with siRNA directed against PHD2 (10 nM) or PHD3 (2.5 nM) at 24 and 48 h. Mock control cells were subjected to transfection procedure without oligonucleotides under the same conditions. After transfection, cells were grown for 24 h and then exposed to normoxic or hypoxic atmosphere for the indicated time. Cells were lysed, and whole cell lysates were extracted as described above.

**Immunofluorescence and Microscopy**—U2OS cells were grown on poly-d-lysine-coated glass coverslips in 24-well dishes overnight. Subconfluent cells were transfected with siRNA as described above and subjected to hypoxia for 6 h, fixed by ice-cold methanol/acetone (1:1) for 10 min on ice, and blocked with 3% bovine serum albumin in PBS. As primary antibody, the mouse monoclonal anti-HIF-1α (diluted 1:50; Transduction Laboratories, San Diego, CA), and as secondary antibody an Alexa-568-conjugated goat anti-mouse IgG (1:400, Molecular Probes, Inc., Eugene, OR) antibody...
was used as described previously (44). Coverslips were
mounted on the slides with Mowiol (Calbiochem). Visualization
was performed with a laser-scanning microscope (LSM
510, Zeiss, Oberkochen, Germany) equipped with a helium/ neon laser. Red fluorescence of Alexa-568 was collected
through a 585-nm long pass filter. The objective lens was a
×63 numerical aperture 1.40 Plan-Apochromat. Fluorescence
intensities were visualized in false colors as indicated by
the color table.

Cell Viability—Toxicity of GSNO was excluded for 250 μM
GSNO as judged from the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-
phenyltetrazolium bromide assay (45).

RESULTS

NO Induces HIF-1α Accumulation and Expression of PHD2 and
-3—Considerable cell-specific and NO donor-dependent
differences have been reported with respect to the effects on
HIF-1α accumulation and PHD activity. We therefore first
determined how 250 μM GSNO affects HIF-1α and PHD protein
levels in U2OS cells. NO maximally induced HIF-1α accumu-
lation under normoxic or hypoxic conditions within 2 h (Fig. 1, A and B). This effect appeared to be transient, since
HIF-1α returned to basal levels within 4 h (Fig. 1A, 4 h). HIF-1α
was maximally induced by hypoxia alone after 4 h and remained
elevated during the course of the experiment. The addition of
NO led to a decrease of HIF-1α, most prominently after 6 h of
incubation (Fig. 1B, 6 h). Thus, NO had a biphasic effect on
HIF-1α induction, with an early increase of HIF-1α followed by
a later inhibition of its accumulation.

PHD1 and PHD2 protein were already found in substantial
amounts under normoxia, whereas PHD3 was hardly detecta-
able (Fig. 1, A and B, 0 h). PHD2 and PHD3 protein levels
were induced by NO under normoxia, particularly at 4 and 6 h for
PHD2 and at 4 h for PHD3 (Fig. 1A), respectively. Under
hypoxia, NO induced PHD2 and PHD3 at 2, 4, and 6 h, whereas
under hypoxia alone induction of PHD2 and PHD3 was
observed at 6 and 8 h (Fig. 1B). PHD1 protein levels were unaf-
fected both by NO or hypoxia (Fig. 1, A and B). Thus, the
observed decrease in HIF-1α accumulation during the late
phase of NO treatment was correlated with an induction of
PHD2 and PHD3 levels (Fig. 1, A and B, 4–8 h). The same
effects on HIF-1α and PHD2 levels were observed in a neuro-
blastoma cell line, SH-SY5Y (data not shown). In addition, sim-
ilar effects were obtained with the NO donor spermine-
NOSOate and DETA-NO (data not shown) but with slightly
slower kinetics according to the longer lasting release of NO
(46).

PHD2 and PHD3 are known HIF-1 target genes (16, 17). We
therefore studied if the NO-dependent induction of HIF-1α in
the early phase of NO treatment is reflected by corresponding
changes of the PHD2 and -3 mRNA levels using quantitative
real time PCR. Transcript levels of both genes were increased
after 2 and 4 h of NO treatment under normoxia or in addition
to hypoxia (Fig. 2, A–D). After 8 h of NO treatment, however,
PHD2 and PHD3 mRNA levels returned to the levels of the
normoxic or hypoxic controls. As with PHD1 protein, PHD1
mRNA levels were neither affected by NO nor by hypoxia.

NO-Induced PHD2 and -3 Expression Is Strictly
HIF-1α-dependent—The early increase and late decrease in
PHD2 and -3 mRNA levels reflect the time course of the HIF-1α
regulation by NO (see Fig. 1). We therefore studied the role of
HIF-1α in the induction of PHD2 and -3 under NO treatment
by suppression of HIF-1α using siRNA. siRNA directed against
HIF-1α completely prevented the induced accumulation of
HIF-1α protein by NO or hypoxia (Fig. 3A), whereas both the
nontarget control siRNA directed against luciferase and the
mock control showed normal induction of HIF-1α. Suppres-
sion of HIF-1α by using siRNA abolished the induction of
PHD2 and -3 protein by NO, which indicates that NO induced
PHD2 and -3 expression, is strictly HIF-1α-dependent in U2OS
cells (Fig. 3B).

PHD Activity is Transiently Inhibited by NO—NO has
been found to inhibit the activity of PHD enzymes in vitro
(28). Indeed, HIF-1α accumulated within the first 2 h of NO
treatment, but this effect disappeared at 4 h under normoxia
and hypoxia (Fig. 1, A and B). To prove a transient inhibition
of PHD activity by NO, the hydroxylation status of HIF-1α
was assessed by Western blot using an antibody against Hyp-
564 (42). For that purpose, proteasomal degradation of
hydroxylated HIF-1α was inhibited by MG132. Treatment

FIGURE 1. NO induces protein levels of HIF-1α, PHD2, and PHD3. U2OS
cells were incubated in the presence (+) or absence (−) of 250 μM GSNO
under normoxic (A) or under hypoxic conditions (B) for the indicated periods
of time. Whole cell lysates were analyzed for the expression of HIF-1α, PHD2,
PHD3, and PHD1 by Western blot analysis. α-Tubulin (α-Tub) was used as a
loading control. Immunoblots shown are representative for at least three
separate experiments.

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with dimethyloxalylglycine, a well known inhibitor of PHD activity (4,12), abolished accumulation of Hyp-564 under normoxia, as expected (Fig. 4A). Likewise, NO decreased Hyp-564 compared with untreated controls under normoxia (Fig. 4B) or inhibited hydroxylation of HIF-1α in addition to hypoxia (Fig. 4C). After 2 h of incubation, Hyp-564 levels were induced under normoxia or at least returned to control levels under hypoxic conditions. Thus, PHD activity had returned after 4 h of NO treatment, indicating that inhibition of PHDs was a transient process. Under normoxia, PHD activity was apparently higher after 4 h of NO treatment, as indicated by increased amounts of Hyp-564.

The transient inhibition of PHDs reflects the release kinetics of NO from GSNO as previously reported (24). We assumed that inhibition of PHDs requires the presence of NO and that the return of PHD activity was most likely due to exhausted NO release from GSNO. To test this hypothesis, we preincubated GSNO in complete medium on cells for 2 h and applied this medium to fresh cells for a further 2-h incubation. In comparison with “fresh” GSNO (plus sign in Fig. 5A), preincubated medium (plus sign with asterisk in Fig. 5A) no longer induced HIF-1α, neither under normoxia nor under hypoxia. Likewise, HIF-1α was reinduced during NO treatment for 4 h (Fig. 5B, 4 h), when fresh GSNO was reapplied for the last 2 h (Fig. 5B, 2 + 2), both under normoxia and hypoxia. The by-products of the NO release from GSNO, GSH and GSSG, did not affect accumulation of HIF-1α, and the NO scavenger carboxyl-PTIO prevented the induction of HIF-1α by NO (data not shown). Thus, the NO-dependent inhibition of PHDs was limited to the time when NO was released from the donor.

NO-induced PHD Protein Levels Promote HIF-1α Degradation—To address the functional consequences of NO-induced PHD2 and -3 expression on HIF-1α in U2OS cells were pretreated with NO for 6 h. At this time point, direct inhibition of PHD activity by NO has already ceased (see Figs. 4 and 5); thus, effects of increased PHD2 and PHD3 protein levels may be expected. Pretreatment with NO under normoxia increased PHD2 and -3 levels and led to delayed accumulation of HIF-1α during a subsequent 2-h hypoxic incubation (Fig. 6A). This effect of PHD2 and -3 induction can also be seen in Fig. 1B by comparing HIF-1α accumulation with or without NO treatment under hypoxia for 6 h.

To analyze the effect of PHD2 and -3 induction on the degradation of HIF-1α upon reoxygenation, cells were pretreated with NO under hypoxic conditions and returned to normoxia (Fig. 6B). Cells pretreated showed a more rapid decrease in HIF-1α levels, which was most prominent after 5 min of reoxygenation. Semiquantitative densitometry of three immunoblots revealed a significantly accelerated degradation of HIF-1α in NO-treated cells compared with untreated controls (Fig. 6C). Thus, higher levels of PHD2 or PHD3 in NO-treated cells significantly decrease cellular HIF-1α. To test for increased PHD activity, we detected the hydroxylation status of HIF-1α under conditions of reoxygenation. We used the antibody against Hyp-564 as described above. Indeed, Hyp-564 was increased in cells pretreated with NO when subjected to reoxygenation for 5 min (Fig. 6D). This clearly indicates that PHD activity is enhanced in cells pretreated with NO under hypoxia.
NO Activates the HIF-1α-PHD2 Autoregulatory Loop

PHD2 but Not PHD3 Contributes to the Regulation of HIF-1α Degradation under NO—To determine whether PHD2 or PHD3 was responsible for the enhanced degradation after NO treatment, we used siRNA to suppress the expression of PHD2 or PHD3 (Fig. 7 and Fig. 8, respectively). siRNA-mediated suppression of PHD2 prevented the NO-induced delay in HIF-1α accumulation under hypoxia (Fig. 7A) as well as the reduced hypoxic accumulation after normoxic preincubation with NO (Fig. 7B). Moreover, accelerated HIF-1α degradation in NO-pretreated cells after 5 min of reoxygenation was completely abolished by knockdown of PHD2 (Fig. 7C). Nontarget control siRNA directed against luciferase affected neither PHD2 nor HIF-1α expression.

In contrast, reduction of PHD3 by siRNA had no impact on NO-induced degradation of HIF-1α under hypoxia (Fig. 8, A–C) or normoxia/hypoxia (Fig. 8D) as shown by immunofluorescence and Western blot analysis. PHD2 and PHD3 were specifically suppressed by their targeting siRNA, since individual PHD protein levels were not reduced by siRNA directed against the other PHD isoform. Because knockdown of PHD2 led to the induction of HIF-1α, PHD3 protein levels were increased both under hypoxia and under NO (Fig. 8, C and D). Taken together, the data indicate that PHD2 is responsible for controlling HIF-1α levels under NO.

DISCUSSION

Several mechanisms have been suggested to explain the opposing effects of NO on HIF-1α accumulation under normoxia and hypoxia. Cellular oxygen concentration, particularly in cell culture systems, results from the ratio of O2 supply to O2 demand. This may cause cellular hypoxia even during incubation in air (21% O2) due to a high oxygen consumption rate and...
a diffusion-limited supply through the medium above the cells (47, 48). NO is a known inhibitor of cytochromes in the respiratory chain and, consequently, of O2 consumption. It has thus been proposed that reduction of HIF-1α by NO results from redistribution of O2 from mitochondrial cytochromes to the cytosol, PHDs are inhibited, which stabilizes HIF-1α (4). Our data indicate that during an early phase of NO treatment, decreased under normoxia (Figs. 1 and 6). Fresh GSNO was added for the last 2 h during NO treatment (2 + 2). Representative immunoblots of at least three independent experiments are shown. α-Tub, α-tubulin.

FIGURE 5. NO release from GSNO is transient. To test for NO release from 250 μM GSNO during 2- or 4-h incubations, GSNO was either preincubated in medium for 2 h or freshly added again after 2 h. HIF-1α accumulation was detected by Western blot analysis of whole cell lysates. A, 250 μM GSNO was preincubated in medium with cells for 2 h under normoxia or hypoxia. U2OS cells were then incubated with (+) or without (−) fresh GSNO or with the preincubated GSNO (+∗). B, U2OS cells were incubated with GSNO under normoxic or hypoxic conditions for 2 or 4 h. Fresh GSNO was added for the last 2 h during NO treatment (2 + 2). Representative immunoblots of at least three independent experiments are shown. α-Tub, α-tubulin.

FIGURE 6. NO-induced PHD levels modulate HIF-1α accumulation and degradation. U2OS cells were pretreated with GSNO for 6 h to induce PHD2 and PHD3 protein levels and tested for HIF-1α accumulation by Western blot analysis. A, cells were preincubated with (+) or without (−) GSNO under normoxia for 6 h and then subjected to hypoxia for 1 and 2 h to induce HIF-1α accumulation. B, cells were incubated in the presence (+) or absence (−) of GSNO under hypoxia for 6 h and afterward subjected to normoxia for the indicated periods of time to induce HIF-1α degradation. C, relative HIF-1α levels of the reoxygenation experiments described above (B) were quantified by densitometry of immunoblots. Data represent the means ± S.D. of three independent experiments.∗, a statistically significant difference between NO-treated samples and controls with p < 0.05 (paired Student’s t test). D, cells were preincubated in presence (+) or absence (−) of GSNO under hypoxia for 6 h and reoxygenated for 5 min. PHD activity was assessed by detection of Hyp-564 by Western blot analysis as described for experiments in Fig. 4. α-Tub, α-tubulin.
when increased amounts of newly synthesized PHD protein decreased HIF-1α (Figs. 1, 4, and 6A).

The time course of PHD inhibition and HIF-1α accumulation by NO closely follows previously determined kinetics of NO release from GSNO (24). These measurements uncovered that NO levels in medium derived from GSNO at 4 h are only 5% of the peak value found 1 h after the addition of GSNO under normoxia. Indeed, when GSNO was preincubated in medium on U2OS cells for 2 h and then transferred to fresh U2OS cells, preincubated GSNO was no longer able to induce HIF-1α during a subsequent 2-h incubation (Fig. 5A). On the other hand, when GSNO was freshly added after 2 h during a 4-h experiment, HIF-1α was induced again. Thus, the NO-dependent accumulation of HIF-1α as a consequence of inhibition of PHD activity was limited to the time period when NO was released from the donor. The lack of HIF-1α accumulation at 4–8 h under normoxia (Fig. 1) supports this notion and already indicates that NO inhibits PHDs in a transient manner.

It is currently unknown how NO acts on PHDs mechanistically. It has been shown for other 2-oxoglutarate-dependent dioxygenases that NO inhibits the enzyme by competing for O₂ at the central iron of the active site (51). Assuming a similar mechanism by which NO inhibits PHDs, O₂ will replace NO again when the NO concentration decreases due to the completed decomposition of GSNO, and PHD activity will resume. Under hypoxia, this process will be limited by the lack of O₂, but induction of newly synthesized PHD2 or PHD3 enzyme that was not inhibited by NO will cause a decrease in HIF-1α levels despite hypoxia (Figs. 1 and 6).

Indeed, several reports suggest that during hypoxic conditions of limited PHD activity, induction of PHD protein level results in higher PHD activity (14, 18, 21, 22). Consequently, the contribution of each PHD strongly depends on the abundance of the enzyme under the particular culture conditions (14). In line with others, we found that PHD2, not PHD3, was most

**FIGURE 7.** PHD2 contributes to regulation of HIF-1α degradation under NO. U2OS cells were transfected with siRNA duplexes to suppress the expression of PHD2 or luciferase (Luc) as a nontargeting control or exposed to transfection reagent alone (Mock) as described under “Experimental Procedures.” Cells were then incubated with (+) or without (−) 250 μM GSNO under hypoxia for 6 h (A), under normoxia for 6 h followed by hypoxia for 2 h (B), or under hypoxia for 6 h followed by reoxygenation for 5 min (C). Accumulation of HIF-1α, PHD2, and α-tubulin was tested by Western blot analysis. Representative immunoblots of at least three independent experiments are shown. α-Tub, α-tubulin.

**FIGURE 8.** PHD3 is not involved in NO-induced HIF-1α degradation. U2OS cells were transfected with siRNA duplexes directed against PHD2 or -3 or were exposed to transfection reagent alone (Mock) as described under “Experimental Procedures.” Cells were incubated with (+) or without (−) 250 μM GSNO under hypoxia for 6 h (A–C) or under normoxia for 6 h followed by 2 h of hypoxia (D). HIF-1α accumulation was detected in cell nuclei by immunofluorescence and visualized in false colors from lowest (blue) to highest fluorescence intensity (red) in A or by Western blot analysis of whole cell lysates (B and C). α-Tub, α-tubulin.
U2OS cells do not express HIF-2α (data not shown) (21), our data fully support the notion that NO modulates the autoregulatory loop of HIF-1α–PHD2 in U2OS cells.

Fig. 9 illustrates our hypothesis of how NO affects HIF-1α and PHD2 in a biphasic manner in U2OS cells. Under normoxic conditions (Fig. 9A), PHD2 hydroxylates HIF-1α, which is directed to proteasomal degradation. Due to the lack of HIF-1α-dependent PHD2 gene expression, the PHD2 protein level remains low (Fig. 9A, Normoxia). In an early phase, NO inhibits PHD activity. Consequently, HIF-1α accumulates, and HIF-1α-dependent induction of PHD2 mRNA expression is followed by higher PHD2 protein levels (Fig. 9A, Early phase). Increased PHD2 protein levels lead to enhanced hydroxylation of HIF-1α, which is completely degraded under normoxia. In turn, PHD2 gene expression returns to basal levels (Fig. 9A, Late phase), which we determined after 8 h of NO treatment (Fig. 2A). Under hypoxic conditions (Fig. 9B), NO further inhibits PHD activity in addition to oxygen deprivation (Fig. 9B, Early phase), thereby enhancing the autoregulatory loop proposed earlier for conditions of hypoxia and reoxygenation (18, 21, 22). Because the effect of NO is transient, whereas hypoxia is ongoing in our experiments, hypoxic levels of PHD2 expression are reached (Fig. 9B, Late phase). In support of this, PHD2 mRNA levels were back to hypoxic levels after 8 h of incubation (Fig. 2B).

In summary, we demonstrate that NO, a mediator of the inflammatory response, can affect O2 sensing by activating the HIF-1α–PHD2 autoregulatory loop just like hypoxia. Initially, in cells that are exposed to NO, PHDs are inhibited and allow accumulation of HIF-1α, but as a consequence, HIF-dependent expression of PHD2 is induced, which results in increased cellular hydroxylation capacity. We identified the induction of PHD2 protein and activity as a mechanism that contributes to NO-induced degradation of HIF-1α.

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