Published in final edited form as:

Biochem Pharmacol. 2011 September 1; 82(5): 485–490. doi:10.1016/j.bcp.2011.05.033.

Hyperoxia attenuates the inhibitory effect of nitric oxide donors on HIF prolyl-4-hydroxylase-2: Implication on discriminative effect of nitric oxide on HIF prolyl-4-hydroxylase-2 and collagen prolyl-4-hydroxylase

Soohwan Yum^a, Jeongyoun Choi^a, Sungchae Hong^a, Myung Hee Park^{a,b}, Jaewon Lee^a, Nam-Chul Ha^a, and Yunjin Jung^{a,*}

^a College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea

^b Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Prolyl 4-hydroxylases (P4Hs), such as collagen prolyl-4-hydroxylases (CPHs) and hypoxia inducible factor prolyl-4-hydroxylases (HPHs), have recently been recognized as promising drug targets for the treatment of fibrotic and ischemic diseases. CPHs and HPHs catalyze identical metabolic reactions, yet lead to quite different physiological consequences, collagen synthesis and the regulation of oxygen homeostasis. Selective modulation of the two enzymes should provide a therapeutic benefit upon pharmacotherapy. In an *in vitro* VHL capture assay, hydroxylation of the 19mer HIF peptide (corresponding to HIF-1 α residues 556–574) by HPH-2 was effectively prevented by nitric oxide (NO) donors, ($\pm$)-S-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione. The NO donors also caused inhibition of HPHs and accumulation of nonhydroxylated HIF-1 α protein in A549 human lung adenocarcinoma cells. Hyperoxia (100% O₂) attenuated both NO donor-induced accumulation of HIF-1 α and inhibition of HPH-mediated hydroxylation. In the presence of a proteasome inhibitor, MG132, the hyperoxia-mediated degradation of HIF-1 α was deterred and hydroxylated HIF-1 α was detected. SNAP, while being an effective inhibitor of proline 4-hydroxylation of HIF-1 α by HPH-2, did not diminish proline hydroxylation of collagen by CPHs. Our data suggest that NO inhibits HPH-2 via competing with dioxygen and that the discriminative effect of NO on CPHs and HPH-2 is attributable to the difference in the affinity of the two enzymes toward dioxygen.

Keywords

Nitric oxide; Hyperoxia; HIF prolyl-4-hydroxylase; Collagen prolyl-4-hydroxylase; Hypoxia inducible factor

1. Introduction

Prolyl 4-hydroxylases (P4Hs) have central roles in the synthesis of collagens and the regulation of oxygen homeostasis [1]. The posttranslational modification, the 4-hydroxylation of a proline residue, carried out by P4Hs, is important for the stability of the collagen triple helix and the degradation of the α subunit of hypoxia-inducible transcription factor (HIF- α) [1]. Vertebrate collagen prolyl-4-hydroxylases (CPHs) are $\alpha_2\beta_2$ tetramers with three isoenzymes differing in their catalytic α subunits [2]. Another P4H family, HIF-P4Hs (HPHs), hydroxylate specific prolines in oxygen degradation domain of HIF- α and regulate von Hippel-Lindau (VHL)-dependent degradation of hydroxylated HIF- α in an oxygen-dependent manner [3-5]. Although CPHs and HPHs catalyze prolyl 4-hydroxylation of different substrates, collagen and HIF- α , respectively, they are mechanistically identical enzymes with a conserved active site. All P4Hs require Fe^{2+} , 2-oxoglutarate, O_2 , and ascorbate to hydroxylate proline(s) in their substrates [1].

While CPHs are regarded as attractive targets for pharmacological inhibition to control excessive collagen accumulation in fibrotic diseases and severe scarring, HPH inhibitors are believed to have beneficial effects in the treatment of diseases such as myocardial infarction, stroke, peripheral vascular disease, diabetes, and severe anemias [1,6]. Although selective modulation of activity of each enzyme holds therapeutic potential for treatment of a spectrum of pathological conditions, agents that selectively inhibit one of the P4Hs have not so far been established. As mentioned above, CPHs and HPHs require iron as a cofactor, 2-ketoglutarate and oxygen as substrates and ascorbate as an iron-reducing agent, to hydroxylate proline residues in its respective substrate, collagen and HIF- α . HPHs and CPHs have similar K_m values for 2-ketoglutarate and a 2-ketoglutarate mimicking agent such as dimethyloxallyl glycine does not exhibit selective inhibition of different prolyl-4-hydroxylases [7]. On the contrary, HPHs have much greater K_m values for another substrate, dioxygen (230–250 μM) than do CPHs (40 μM) [8], suggesting that a dioxygen-mimicking agent is likely to distinguish the two enzymes.

Nitric oxide (NO) was reported to inhibit HPHs, although its inhibitory mechanism remains controversial [9,10]. For catalytic hydroxylation of HIF-1 α , dioxygen (O_2) binds to Fe(II) in the active site of HPHs [11]. NO is a molecular mimic for O_2 binding as an Fe(II) ligand. Indeed, high resolution structures of globins and heme oxygenase complexed with O_2 or with NO show very similar binding geometries and local interactions in the heme pocket [12,13]. Therefore, NO-mediated inhibition of HPHs is likely due to its competition with dioxygen, which raises the possibility of discriminative effect of NO on HPHs and CPHs. Our data provide strong evidence that NO inhibits HPH-2 directly through its competition with dioxygen binding and that NO exerts a selective inhibition of HPH-2 and HIF-1 α prolyl hydroxylation without inhibiting collagen prolyl hydroxylation by CPHs, in A549 human lung adenocarcinoma cells.

2. Materials and methods

2.1. Chemicals, plasmids and cell culture

S-nitrosoglutathione (GSNO), ($\pm$)-S-nitroso-N-acetylpenicillamine (SNAP), 1, 10-phenanthroline (phenanthroline), β -aminopropionitrile and ciclopirox were purchased from Sigma (St. Louis, MO). All other chemicals were reagent-grade, commercially available products. HPH-2 plasmid was kindly provided by S. McKnight (University of Texas Medical Center, Dallas, TX) and Flag-VHL plasmid was a kind gift from Dr. Len Neckers (National Cancer Institute, NIH, MD). Biotinylated HIF-peptide (Biotin-Ahx-DLDLEALAPYIPADDDFQL, WT-HIF), HIF-peptide with hydroxyproline (Biotin-Ahx-DLDLEALA(-HyP)YIPADDDFQL, HIF-P546Hyp) and HIF-peptide with 564 alanine (Biotin-Ahx-DLDLEALAAAYIPADDDFQL, HIF-P546A) corresponding to HIF residues 556–574 were synthesized (Peptron, Daejeon, Republic of Korea). Streptavidin beads were purchased from Pierce Immunopure (Rockford, IL). A549 human lung carcinoma cells (ATCC, Manassas, VA), which are commonly used for experiments to see hyperoxia effects, were incubated in 5% CO₂ at 37 °C in a humidified incubator (Forma, Asheville, NC). The cells were grown in DMEM medium (Hyclone, Logan, Utah) supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin (Hyclone) and subcultured every 5–7 days by harvesting in trypsin (Invitrogen, Carlsbad, CA) no more than 20 times from stock originally designated at pass 70. Cells were cultured for 24 h in 35-mm tissue culture plates (Sarstedt, Nümbrecht, Germany) at 1.5×10^6 cells/ml and subsequently exposed to normoxia (21% O₂ and 5% CO₂) or hyperoxia (100% O₂). The hyperoxia group was placed in a hyperoxia/hypoxia chamber (Billups-Rothenberg, Inc. Del Mar, CA) in a cell culture incubator (Forma). When indicated, an NO donor, SNAP or GSNO, was added just before exposure to hyperoxia.

2.2. Immunoblot analysis

A549 cells were lysed and nuclear or whole cell extracts were prepared as described previously [14]. In some experiments, cells were lysed in the presence of an iron chelator, phenanthroline (200 μ M), to prevent HIF-1 α hydroxylation during the preparation of cellular lysates. Protein concentration in the supernatants was determined by the BCA method. The cellular extracts were electrophoretically separated using 7.5 or 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, NH) and hypoxia-inducible factor-1 α (HIF-1 α) and hydroxylated HIF-1 α were detected in nuclear extracts (30–40 μ g) using a monoclonal anti-HIF-1 α antibody (BD Biosciences Pharmingen, San Jose, CA) and a monoclonal anti-hydroxylated HIF-1 α antibody (Cell Signaling Technology, Beverly, MA). Peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:2000. Signals were visualized using the SuperSignal chemiluminescence substrate (Pierce, Rockford, IL). Experiments were performed at least in duplicate and normalized with antibodies to topoisomerase II (Santa Cruz Biotechnology).

2.3. Collagen prolyl-4-hydroxylase assay

For determination of collagen proline 4-hydroxylation, A549 cells were plated in 60 mm dishes. After cell attachment, ascorbic acid (50 μ g/ml), β -aminopropionitrile (30 μ g/ml) and

the test compounds were added along with [^3H]proline (20 $\mu\text{Ci/ml}$) (Amersham, Piscataway, NJ). After a 18 h incubation, labeled proteins (including collagen) released into the medium were precipitated with 10% TCA. After washing 3 times with 5% TCA solution, precipitated proteins were hydrolyzed in 6 N HCl at 115 $^{\circ}\text{C}$ for 24 h. Unlabeled hydroxyproline was added to each sample as a carrier. Radioactive proline and hydroxyproline were separated by TLC on silica gel-coated glass plates (Whatman, Clifton, NJ) in 75% phenol (phenol:water, 75:25, w/w) containing 0.2 mg/ml NaCN. The positions of proline and hydroxyproline were visualized by the ninhydrin reaction. Hydroxyproline separated from proline with R_f values of 0.45 and 0.58, respectively. The silica gel in the hydroxyproline and proline areas was scraped off and eluted with 0.5 ml of water, and the radioactivity in each spot was counted using a Beckman (Fullerton, CA) liquid scintillation spectrometer. The degree of proline hydroxylation was estimated from the ratio of [^3H]hydroxyproline to the total radioactivity in [^3H]proline plus [^3H]hydroxyproline.

2.4. HIF prolyl-4-hydroxylase assay (in vitro VHL capture assay)

Biotinylated wild type or proline-hydroxylated peptides (corresponding to HIF-1 α residues 556–574) were dissolved in sterile water (500 $\mu\text{g/ml}$) and incubated with streptavidin beads (Pierce ImmunoPure, Rockford, IL) at 4 $^{\circ}\text{C}$ for 2 h. The beads were washed twice with VHL binding buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40) and three times with reaction buffer (20 mM Tris pH 7.5, 5 mM KCl, 1.5 mM MgCl_2). For each condition, 2 μg peptide/20 μl beads was aliquoted into separate tubes and the reaction buffer was added, along with cofactors (100 μM 2-ketoglutaric acid, 100 μM L-ascorbic acid, 50 μM ferrous chloride). The beads and HPH cofactors were mixed at room temperature for 15 min in the reaction buffer. Prior to this incubation, any inhibitors or competing factors were added to the appropriate tubes. HIF prolyl hydroxylase-2 protein and Flag-VHL protein were produced by separate *in vitro* translated (IVT) reactions (Promega, Madison, WI) [15] using HPH-2 plasmid and Flag-VHL plasmid. A 5 μl aliquot of IVT HPH-2 was added to the bead-peptide mixture for 1 h at 30 $^{\circ}\text{C}$. Subsequently, the beads were washed with VHL binding buffer and 10 μl Flag-VHL IVT was added to the beads overnight at 4 $^{\circ}\text{C}$. The beads were washed, SDS sample buffer was added, the samples were boiled, subjected to SDS-PAGE, and resultant blots were probed for Flag [14]. To perform the VHL capture assay under hyperoxia, the air in a microtube ready for the HIF hydroxylation reaction was replaced with oxygen gas and the tube was sealed tightly and placed in a 50 ml tube filled with oxygen gas.

3. Results

3.1. Nitric oxide donors inhibit HIF prolyl hydroxylase-2

It was previously reported that NO donors inhibit HPHs, based on results from a cell based VHL capture assay in which hydroxylation of cellular HIF-1 α was determined by co-immunoprecipitation of ^{35}S -labeled VHL with the hydroxylated HIF-1 α protein [9]. However, a recent paper suggests that nitric oxide (NO)-mediated inhibition of VHL recruitment to HIF-1 α occurs by nitrosylation of cysteine 520 in HIF-1 α , rather than inhibition of HIF-1 α hydroxylation by HPH-2 [10]. Since cellular HIF-1 α used in the cell-based VHL capture assay contains both cysteine 520 (for nitrosylation) and proline 402 and

564 (for hydroxylation), further verification is required to determine whether NO inhibits HPH activity directly. To clarify this point, we carried out an *in vitro* VHL capture assay using a substrate of HPH-2, 19mer HIF peptide (Biotin-Ahx-DLDLEALAP564YIPADDDFQL) not containing cysteine 520 (WT-HIF), in order to rule out an involvement of the cysteine nitrosylation in VHL association with the HIF peptide. Nitric oxide donors SNAP and GSNO were added to the *in vitro* VHL capture assay using the biotinylated WT-HIF peptide bound to avidin beads. As shown in Fig. 1A, the association of Flag-VHL with the WT-HIF peptide was undetectable in the absence of exogenously added cofactors (lane 1). When the required cofactors for HPH-2 were added, the association between the WT-HIF peptide and Flag-VHL is markedly enhanced (lane 2). Addition of 0.2 mM SNAP significantly reduced the association between the WT-HIF peptide and VHL, and at 0.4 mM, SNAP completely abrogated the interaction. Another nitric oxide donor, GSNO, also reduced the association between the WT-HIF peptide and VHL, but GSNO was less potent than SNAP. In contrast to the WT-HIF peptide, a mutated HIF peptide (HIF-P564A, a peptide in which the proline 564 was substituted with alanine) failed to bind VHL under any circumstances (Fig. 1A, lanes 9–12). To exclude a possibility that an amino acid residue(s) in the WT-HIF peptide was modified by NO donors leading to inhibition of VHL recruitment, hydroxylated HIF peptide (HIF-P546Hyp) (Biotin-Ahx-DLDLEALA(HyP)YIPADDDFQL) was treated with the NO donors and subsequently Flag-VHL was added, followed by detection of recruited VHL. As shown in Fig. 1B (left panel), nitric oxide donors did not impair the ability of VHL to associate with the hydroxylated-HIF peptide. Thus, our data suggest that NO is an inhibitor of HPH, rather than a direct inhibitor of HIF-1 α /VHL association.

To confirm this in cells, immunoblot analysis for hydroxylated cellular HIF-1 α was performed after treatment with NO donors (Fig. 1C). An iron chelator, ciclopirox, was used as a control inhibitor of HPH-2. Cells treated with or without the NO donors were lysed to obtain nuclear extracts and the levels of hydroxylated HIF-1 α in the nuclear extract were compared. HIF-1 α protein was induced in all cells treated with SNAP (Fig. 1C, lanes 2 and 4), GSNO (lanes 6 and 8) and ciclopirox (lanes 10 and 12), suggesting stabilization of HIF-1 α in these cells. However, hydroxylated HIF-1 α was not detected when prolyl hydroxylation during lysis was prevented by inclusion of a HPH inhibitor, phenanthroline, in the lysis buffer (lanes 4 and 8). But if phenanthroline is omitted, strong signals of hydroxylated HIF-1 α were observed in the lysates of NO-donor-treated cells (Fig. 1C, lanes 2 and 6). Thus, this observation suggests that although NO-induced HIF-1 α was in the nonhydroxylated state, the HIF-1 α was hydroxylated during lysis probably due to the dissociation of NO from HPHs. To confirm this, the same experiment was done with a NO donor, SNAP, instead of phenanthroline. SNAP was added to the lysis buffer followed by cell lysis and the hydroxylation status of NO donor-induced HIF-1 α was monitored. As expected, SNAP blocked HIF-1 α hydroxylation exhibiting an effect similar to that of phenanthroline (Fig. 1D). These results indicate that NO donors inhibit cellular HPHs resulting in stabilization and accumulation of HIF-1 α protein in these cells. On the other hand, the control, ciclopirox-induced HIF-1 α was kept in a nonhydroxylated state in any lysis conditions (lanes 10 and 12), probably because the interaction between the iron chelator and HPHs was strong enough to prevent their dissociation during cell lysis.

3.2. Hyperoxia attenuates the inhibitory effect of NO on HIF-1 α prolyl hydroxylation

Although our data indicate that NO inhibits HPH activity, the inhibitory mechanism is still elusive. Since NO is a molecular mimic for O₂ binding as an Fe(II) ligand and HPHs are dioxygenases with non-heme iron in their active sites, we hypothesized that NO-mediated HPH inhibition occurred by its competition with dioxygen. To test this possibility, NO donors were subjected to an *in vitro* VHL capture assay under hyperoxia (100% oxygen) and normoxia (21% oxygen), and VHL binding was compared. Ciclopirox and caffeic acid phenethyl ester (CAPE), a natural product with HPH inhibitory activity [16], were used as controls. Hyperoxia did not substantially increase VHL binding in the control HPH reactions without NO donors (Fig. 2A, compare lanes 4 with 1, and 10 with 7). However, VHL binding attenuated by NO donors was partially but definitely reversed by hyperoxia (Fig. 2A, compare lanes 5 with 2, 6 with 3, 11 with 8, and 12 with 9), suggesting that HPH inhibition by NO donors is associated with competition with dioxygen. The effect was more evident for SNAP than GSNO. No hyperoxia-mediated attenuation of inhibition of HPH-2 was observed with the control HPH inhibitors, ciclopirox and CAPE (Fig. 2A, bottom panel).

To confirm the hyperoxia effect in cells, A549 cells were treated with NO donors or the known HPH inhibitors under hyperoxia or normoxia and the level of the nuclear HIF-1 α was compared. As shown in Fig. 2B, the level of HIF-1 α protein induced by NO donors was reduced significantly by hyperoxia (top panel, compare lane 5 with 2, lane 6 with 3, lane 11 with 8, lane 12 with 9), while HIF-1 α induction by CAPE and ciclopirox was not affected substantially by the concentration of oxygen (Fig. 2B, bottom panel). This finding implies that hyperoxia counteracted NO donor-mediated suppression of HIF-1 α hydroxylation and degradation. To verify whether the HIF-1 α degradation by hyperoxia occurred by reactivation of HPHs, the same experiment was carried out in the presence of a proteasome inhibitor MG132. As shown in Fig. 2C, SNAP-mediated induction of HIF-1 α was reduced by hyperoxia (compare lane 5 with 2) and this effect was overcome by MG132 (compare lane 6 with 5). Moreover, hydroxylated HIF-1 α was detected in the HIF-1 α protein in the presence of MG132, while hardly detectible without MG132 (compare lane 3 with 2, and lane 6 with 5). Although hydroxylated HIF-1 α was also detected upon treatment with SNAP/MG132 under normoxia, its level was much lower than that obtained from the hyperoxia counterpart (Fig. 2C, compare lane 3 with 6). On the other hand, MG132-mediated accumulation of hydroxylated HIF-1 α was not affected by the concentration of oxygen (Fig. 2C, compare lane 8 with 10).

3.3. SNAP does not inhibit collagen prolyl hydroxylase

CPHs and HPHs, alpha-ketoglutarate- and Fe(II)-dependent dioxygenases, perform the same catalytic reaction (hydroxylation of proline), but on different substrates, collagen and HIF- α , respectively. It is known that the two enzymes have greatly different K_m values for dioxygen (40 μ M for CPH and 230–250 μ M for HPHs), suggesting a possibility that NO exhibits selectivity in inhibiting the two enzymes. To test this hypothesis, the effects of SNAP on collagen prolyl hydroxylation were determined in A549 cells. Cells were cultured with ¹³C-labeled proline in the absence or presence of SNAP (0.3–1.0 mM) and radiolabeled collagen released into the medium was analyzed for hydroxyproline content after acid hydrolysis and

TLC separation. The effects were also measured with known common inhibitors of HPHs, CAPE and ciclopirox [16,17]. As shown in Fig. 3, SNAP, at a concentration range that greatly inhibited HPH-2 in the same cells, did not diminish formation of hydroxyproline in collagen. On the contrary, SNAP seemed to enhance the collagen hydroxylation at high concentrations. The control inhibitors caused marked inhibition of collagen prolyl hydroxylation. These findings clearly demonstrate that SNAP exerts selective inhibition of HPH-2 without suppressing collagen prolyl hydroxylation.

4. Discussion

In this report, we provide strong evidence that nitric oxide donors inhibit HPH-2, but not CPHs, in A549 cells and that NO inhibition of HPH-2 is attenuated significantly by hyperoxia. In view of the widely different K_m values of the two enzymes for dioxygen and also of a similar mode of binding of dioxygen and NO to heme-iron proteins, our data support the notion that competition of NO with dioxygen is relevant to the discriminative effect of NO against HPH-2 and CPHs.

Our data further suggest that nitric oxide directly inhibits HPHs to block hydroxylation of HIF-1 α and to stabilize it. This argument is supported by the suppression of HPH-2-mediated hydroxylation of the biotinylated HIF-1 α peptide (WT-HIF, corresponding to HIF residues 556–574 without 520 cysteine residue) by nitric oxide donors, SNAP and GSNO *in vitro* as indicated by the dramatic decrease of VHL binding (Fig. 1A). Besides direct inhibition of the HPH reaction, it is plausible that NO directly inhibits VHL binding to the HIF-1 α irrespective of Pro564 hydroxylation. However, this possibility can be ruled out based on the following observations: (i) Hydroxylated HIF-1 α peptide binding to VHL was not affected in the presence of SNAP or GSNO (Fig. 1B), (ii) A HIF-1 α peptide with alanine in the place of proline 564 failed to bind to VHL under any circumstances. These *in vitro* results were confirmed by cellular experiments that showed the NO donor-induced accumulation of nonhydroxylated HIF-1 α (Fig. 1C and D).

Our conclusion is in disagreement with that of a recent paper by Park et al. [10], which suggests that nitric oxide (NO) inhibition of VHL recruitment occurs by nitrosylation of the 520 cysteine in HIF-1 α , and that SNAP does not directly inhibit hydroxylation of HIF peptide by HPH-2. Although this discrepancy seems difficult to reconcile, it is possible that the truncated recombinant HPH-2 (amino acids 184–418) used in that paper may be less susceptible to NO than a full length HPH-2. Unlike a full length intact HPH-2 that shows much less activity in the absence of ferrous iron [18], the truncated HPH-2 was not affected by iron concentration, which raises a possibility that the catalytic activity (involving iron) and/or K_m value (for dioxygen) of the truncated HPH-2 could also be altered from those of a full length HPH-2.

Our data suggest that NO inhibition of HPH-2 occurred by its competition with dioxygen binding. This argument is based on the observations that hyperoxia significantly attenuated the NO-mediated HIF-1 α induction and restored VHL binding suppressed by NO donors. The specificity of the hyperoxia effect was evident in that hyperoxia had no effect on an iron chelator- or CAPE-mediated induction of HIF-1 α and suppression of VHL binding.

Moreover, hyperoxia-mediated HIF-1 α degradation seems to occur by reactivation of HPHs, because the level of hydroxylated HIF-1 α of the NO-donor treated cells was much higher under hyperoxia than under normoxia in the presence of a proteasome inhibitor MG132 (Fig. 2C, lanes 6 vs 3).

Although SNAP effectively inhibited HPH-2, it did not impair CPHs activity as shown in Fig. 3. On the contrary, the known HPH inhibitors, ciclopirox and CAPE, drastically decreased the collagen hydroxyproline level. At high concentrations (>0.5 mM), SNAP was found to elevate the collagen hydroxyproline level. Dooley et al. reported that another NO donor, DETA-NO, lowers activity of prolyl-4 hydroxylase to 76% of the control in fibroblasts [19], and suggested that this NO donor reduces collagen synthesis by impairing proline hydroxylation. On the other hand, there is a contrasting report that NO has a stimulatory effect on collagen synthesis in rat dermal fibroblasts [20], consistent with our findings. The discrepancy in NO effects on CPHs and collagen synthesis may be due to unknown variables in the different biological systems.

The two different prolyl-4 hydroxylases, HPHs and CPHs, have different K_m values for dioxygen, and HPHs have much lower affinity for dioxygen. Consistent with this difference in kinetic parameters, HPHs display high sensitivity to hypoxic conditions, whereas CPHs seem to be fully functional under hypoxia [21,22]. HPHs are able to elicit their maximal activity under normoxia (21% oxygen) but lose their enzymatic activity as the oxygen concentration decreases, conferring their function as oxygen sensors [23]. Considering these facts, selective inhibition of HPH-2 by NO competition of dioxygen binding may provide a rational explanation for the discriminative effect of NO toward CPHs and HPH-2. Thus, NO donors might be applicable for the treatment of diseases such as ischemic disorders in which HIF-1 activation is beneficial, without side effects resulting from the inhibition of CPHs.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No: 2009-0071594) and a grant of the Korea Healthcare technology R and D Project (A080640), Ministry for Health, Welfare and Family Affairs, Republic of Korea.

Abbreviations

NO	Nitric oxide
GSNO	S-nitrosoglutathione
SNAP	($\pm$)-S-nitroso-N-acetylpenicillamine
HIF-1α	Hypoxia inducible factor-1 alpha
HPH	HIF prolyl-4-hydroxylase
VHL	von Hippel-Lindau
IVT	<i>In vitro</i> translated
CAPE	Caffeic acid phenethyl ester

P4Hs	Prolyl 4-hydroxylases
CPHs	Collagen prolyl-4-hydroxylases

References

1. Myllyharju J. Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and regulation of the response to hypoxia, and their roles as treatment targets. *Ann Med.* 2008; 40:402–17. [PubMed: 19160570]
2. Myllyharju J. Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol.* 2003; 22:15–24. [PubMed: 12714038]
3. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science.* 2001; 292:468–72. [PubMed: 11292861]
4. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science.* 2001; 294:1337–40. [PubMed: 11598268]
5. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science.* 2001; 292:464–8. [PubMed: 11292862]
6. Hewitson KS, Schofield CJ. The HIF pathway as a therapeutic target. *Drug Discov Today.* 2004; 9:704–11. [PubMed: 15341784]
7. Song YR, You SJ, Lee YM, Chin HJ, Chae DW, Oh YK, et al. Activation of hypoxia-inducible factor attenuates renal injury in rat remnant kidney. *Nephrol Dial Transplant.* 2010; 25:77–85. [PubMed: 19737871]
8. Hirsila M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J Biol Chem.* 2003; 278:30772–80. [PubMed: 12788921]
9. Metzen E, Zhou J, Jelkmann W, Fandrey J, Brune B. Nitric oxide impairs normoxic degradation of HIF-1 α by inhibition of prolyl hydroxylases. *Mol Biol Cell.* 2003; 14:3470–81. [PubMed: 12925778]
10. Park YK, Ahn DR, Oh M, Lee T, Yang EG, Son M, et al. Nitric oxide donor, ($\pm$)-S-nitroso-N-acetylpenicillamine, stabilizes transactive hypoxia-inducible factor-1 α by inhibiting von Hippel-Lindau recruitment and asparagine hydroxylation. *Mol Pharmacol.* 2008; 74:236–45. [PubMed: 18426857]
11. Bruegge K, Jelkmann W, Metzen E. Hydroxylation of hypoxia-inducible transcription factors and chemical compounds targeting the HIF- α hydroxylases. *Curr Med Chem.* 2007; 14:1853–62. [PubMed: 17627521]
12. Friedman J, Lad L, Deshmukh R, Li H, Wilks A, Poulos TL. Crystal structures of the NO- and CO-bound heme oxygenase from *Neisseria meningitidis*. Implications for O₂ activation. *J Biol Chem.* 2003; 278:34654–9. [PubMed: 12819228]
13. Draghi F, Miele AE, Travaglini-Allocatelli C, Vallone B, Brunori M, Gibson QH, et al. Controlling ligand binding in myoglobin by mutagenesis. *J Biol Chem.* 2002; 277:7509–19. [PubMed: 11744723]
14. Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, Chung YL, et al. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell.* 2005; 8:143–53. [PubMed: 16098467]
15. Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM. Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 α -degradative pathway. *J Biol Chem.* 2002; 277:29936–44. [PubMed: 12052835]
16. Choi D, Han J, Lee Y, Choi J, Han S, Hong S, et al. Caffeic acid phenethyl ester is a potent inhibitor of HIF prolyl hydroxylase: structural analysis and pharmacological implication. *J Nutr Biochem.* 2010; 21:809–17. [PubMed: 19740641]

17. Linden T, Katschinski DM, Eckhardt K, Scheid A, Pagel H, Wenger RH. The antimycotic ciclopirox olamine induces HIF-1 α stability, VEGF expression, and angiogenesis. *FASEB J*. 2003; 17:761–3. [PubMed: 12594177]
18. Ivan M, Haberberger T, Gervasi DC, Michelson KS, Gunzler V, Kondo K, et al. Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. *Proc Natl Acad Sci USA*. 2002; 99:13459–64. [PubMed: 12351678]
19. Dooley A, Gao B, Shi-Wen X, Abraham DJ, Black CM, Jacobs M, et al. Effect of nitric oxide and peroxynitrite on type I collagen synthesis in normal and scleroderma dermal fibroblasts. *Free Radic Biol Med*. 2007; 43:253–64. [PubMed: 17603934]
20. Witte MB, Thornton FJ, Efron DT, Barbul A. Enhancement of fibroblast collagen synthesis by nitric oxide. *Nitric Oxide*. 2000; 4:572–82. [PubMed: 11139365]
21. Takahashi Y, Takahashi S, Shiga Y, Yoshimi T, Miura T. Hypoxic induction of prolyl 4-hydroxylase α (I) in cultured cells. *J Biol Chem*. 2000; 275:14139–46. [PubMed: 10799490]
22. Levene CI, Bates CJ. The effect of hypoxia on collagen synthesis in cultured 3T6 fibroblasts and its relationship to the mode of action of ascorbate. *Biochim Biophys Acta*. 1976; 444:446–52. [PubMed: 183826]
23. Mole DR, Maxwell PH, Pugh CW, Ratcliffe PJ. Regulation of HIF by the von Hippel-Lindau tumour suppressor: implications for cellular oxygen sensing. *IUBMB Life*. 2001; 52:43–7. [PubMed: 11795592]

Fig. 1.

Nitric oxide donors inhibit HIF prolyl hydroxylase-2. (A) The *in vitro* VHL capture assay was performed as described in Section 2, in the presence or absence of cofactors (50 μM ferrous chloride, 100 μM sodium 2-ketoglutarate, 100 μM sodium ascorbate) and the indicated concentrations of a NO donor, SNAP or GSNO, and resultant blots were probed for Flag (VHL). Data from lanes 1 to 8 and lanes 9 to 12 were obtained by using WT-HIF peptide and HIF-P546A peptide, respectively. (B) The same assay was repeated utilizing a chemically hydroxylated peptide and a NO donor, SNAP or GSNO (1 mM). (C) A549 cells were treated with a NO donor, SNAP or GSNO, or an iron chelator, ciclopirox, at the indicated concentrations and were lysed 4 h later. The cell lysis was done in the presence or absence of a HPH inhibitor, phenanthroline. Blots were probed with an anti-HIF-1α antibody or an anti-hydroxylated HIF-1α antibody. (D) A549 cells were treated with a NO donor, SNAP (1 mM), and were lysed 4 h later. The cell lysis was done in the presence or absence of SNAP (1 mM). Blots were probed with an anti-HIF-1α antibody or an anti-hydroxylated HIF-1α antibody.

Fig. 2.

Hyperoxia attenuates the inhibitory effect of NO donors on HIF prolyl hydroxylase-2. (A) The *in vitro* VHL capture assay under hyperoxic or normoxic condition was performed as in Fig. 1A in the presence of the indicated concentrations of a NO donor, SNAP or GSNO (top panel), CAPE or ciclopriox (bottom panel). (B) A549 cells, under hyperoxia or normoxia, were treated with a NO donor, SNAP or GSNO (top panel), CAPE or an iron chelator ciclopriox (bottom panel), and were lysed 4 hr later.

Nuclear HIF-1α was detected using an anti-HIF-1α antibody and Topo II as a control. (C) A549 cells, under hyperoxia or normoxia, were treated with a NO donor SNAP (1 mM) in the presence of a proteasome inhibitor MG132 (10 μM) and were lysed 4 h later. The cell lysis was done in the presence of a HPH inhibitor, phenanthroline. Blots were probed with an anti-HIF-1α antibody or an anti-hydroxylated HIF-1α antibody.

Fig. 3.

The nitric oxide donor, SNAP, does not inhibit collagen prolyl hydroxylation. A549 cells were plated in 60 mm dishes. After cell attachment, ascorbic acid (50 μg/ml), β-aminopropionitrile (30 μg/ml) and either SNAP, CAPE or ciclopriox at the indicated concentrations were added along with [³H]proline (20 μCi/ml). After 18 h incubation, labeled proteins (including collagen) released into the medium was subjected to precipitation, acid hydrolysis, TLC separation and scintillation counting to determine the degree of proline hydroxylation as described under Section 2.