0026-895X/08/7401-236–245$20.00
MOLECULAR PHARMACOLOGY
Vol. 74, No. 1
45278/3355787
Mol Pharmacol 74:236–245, 2008
Printed in U.S.A.

Nitric Oxide Donor, (±)-*S*-Nitroso-*N*-acetylpenicillamine, Stabilizes Transactive Hypoxia-Inducible Factor-1α by Inhibiting von Hippel-Lindau Recruitment and Asparagine Hydroxylation[S]

Young-Kwon Park, Dae-Ro Ahn, Myoungsuk Oh, Taekyoung Lee, Eun Gyeong Yang, Miwon Son, and Hyunsung Park

Department of Life Science, University of Seoul, Seoul, Korea (Y.-K.P., M.O., T.L., H.P.); Life Science Division, Korea Institute of Science and Technology, Seoul, Korea (D.-R.A., E.G.Y.); and Research Labs of Dong-A Pharm. Co. Ltd., Kyunggi-do, Korea (M.S.)

Received January 14, 2008; accepted April 21, 2008

ABSTRACT

We have confirmed that the NO donor (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP) stabilizes the transactive form of hypoxia-inducible factor-1α (HIF-1α), leading to the induction of HIF-1α target genes such as vascular endothelial growth factor and carbonic anhydrase 9. Activation of HIF-1α should require inhibition of the dual system that keeps it inactive. One is ubiquitination, which is triggered by hydroxylation of HIF-1α-proline and the subsequent binding of E3 ubiquitin ligase, the von Hippel Lindau (VHL) protein. The other is hydroxylation of HIF-1α-asparagine, which reduces the affinity of HIF-1α for its coactivator, cAMP responsive element binding protein/p300. We examined the effects of the NO donor SNAP on proline and asparagine hydroxylation of HIF-1α peptides by measuring the activities of the corresponding enzymes, HIF-1α-specific proline hydroxylase 2 (PHD2) and the HIF-1α-specific asparagine hydroxylase, designated factor inhibiting HIF-1α (FIH-1), respectively. We found that the SNAP did not prevent PHD2 from hydroxylating the proline of HIF-1α. Instead, it blocked the interaction between VHL and the proline-hydroxylated HIF-1α, but only when the reducing agents Fe(II) and vitamin C were limiting. The fact that the absence of cysteine 520 of HIF-1α abolishes its responsiveness to SNAP suggests that this residue mediates the inhibition by SNAP of the interaction between VHL and HIF-1α, presumably by *S*-nitrosylation of HIF-1α. Unlike PHD2, asparagine hydroxylation by FIH-1 was directly inhibited by SNAP, but again only when reducing agents were limiting. Substitution of cysteine 800 of HIF-1α with alanine failed to reverse the inhibitory effects of SNAP on asparagine hydroxylation, implying that FIH-1, not its substrate HIF-1α, is inhibited by SNAP.

This study was supported by grant 2004-01969 from the Neurobiology Research Program, grant R01-2007-000-11672-0 from the Korea Science and Engineering Foundation, by grant CBM1-B113-001-1-0-0 from the center for Biological Modulators of the 21st Century Frontier R&D Program of the Ministry of Science and Technology (to H.P.), and by grant from the Korea Institute of Science and Technology (to E.G.Y.). Y.-K.P. was supported by a Brain Korea 21 Research Fellowship from the Ministry of Education and Human Resources Development and by a Seoul Science Fellowship.

All experiments reported in this article were repeated with similar results.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.108.045278.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

Hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor, is a master transcription activator responsible for gene induction under hypoxic conditions (Semenza, 2000). It is composed of an α subunit and a β subunit. HIF-1α is ubiquitinated and rapidly degraded under normoxic conditions (Jaakkola et al., 2001). The stability and activity of the α subunit of HIF-1 are regulated by post-translational modification, specifically by hydroxylation. Proline residues 402 and 564 of the oxygen-dependent degradation domain (ODD; amino acids 401–603 of human HIF-1α) are hydroxylated, mainly by HIF-1α-specific proly-4-hydroxylase 2 (PHD2)

ABBREVIATIONS: HIF-1α, hypoxia-inducible factor-1α; α-KG, α-ketoglutarate; CA9, carbonic anhydrase 9; CBP, cAMP-responsive element binding protein; FIH-1, factor inhibiting hypoxia-inducible factor-1α; HRE, hypoxia-responsive element; ODD, oxygen-dependent degradation domain of hypoxia-inducible factor-1α; PHD, hypoxia-inducible factor-1α-specific prolyl hydroxylase; ROS, reactive oxygen species; SNAP, (±)-*S*-nitroso-*N*-acetylpenicillamine; spermine NONOate, *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine; TPEN, *N,N,N′,N′*-tetrakis (2-pyridylmethyl) ethylenediamine; VHL, von Hippel-Lindau; VEGF, vascular endothelial growth factor; HSP70, 70-kDa heat shock protein; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; NOC18, 2,2′-(hydroxynitrosohydrazino) bis-ethanamine; GSNO, *S*-nitrosoglutathione; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; HA, hemagglutinin; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

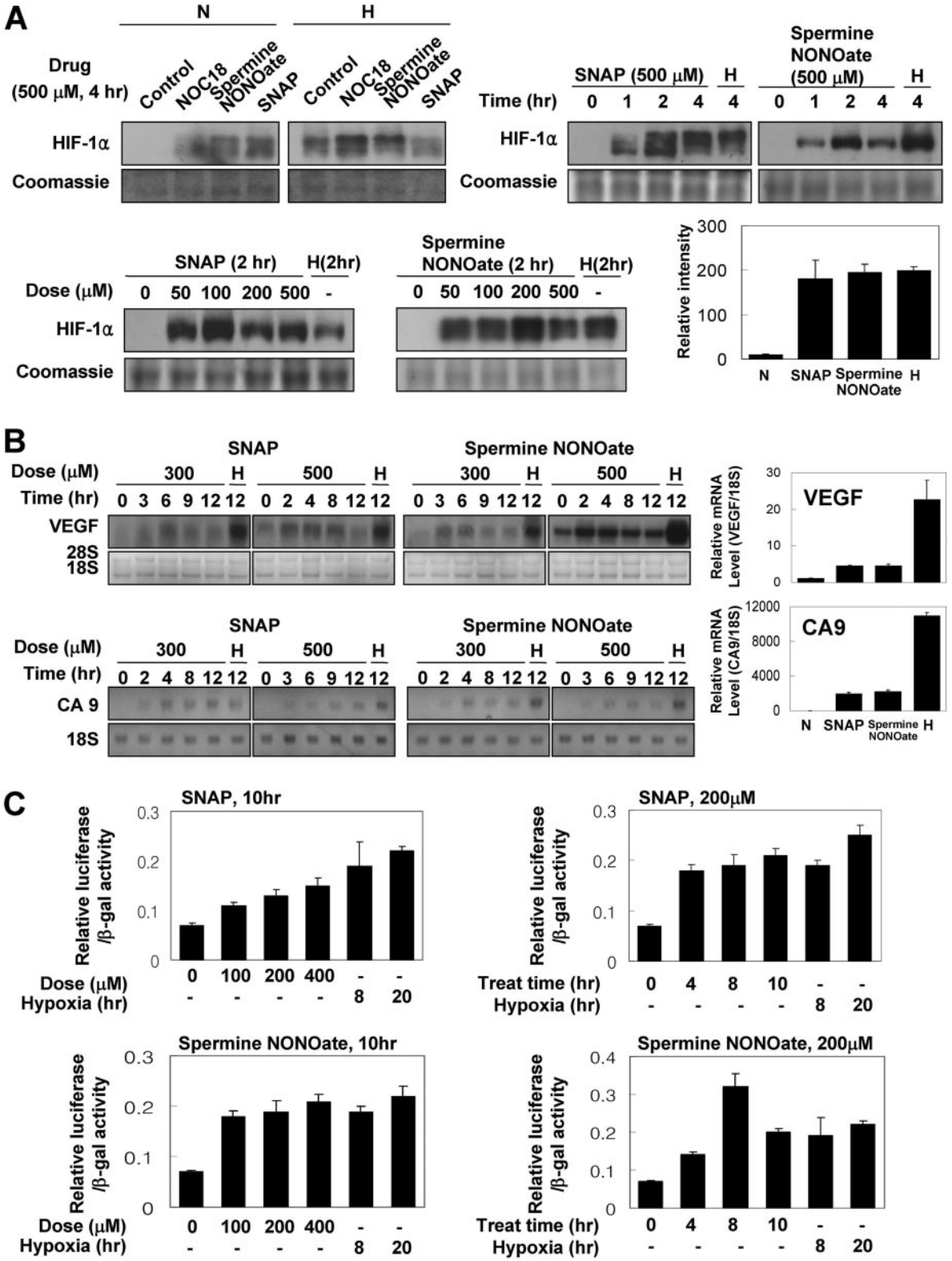

Fig. 1. Effects of NO-related drugs on HIF-1α and its target genes. HeLa cells were treated with the NO donor drugs, NOC18, spermine NONOate, SNAP, and GSNO at different times and doses. A, the protein level of HIF-1α in each sample was visualized by Western blot analysis using anti-HIF-1α antibody (Choi et al., 2006b). The SDS-PAGE gels were stained with Coomassie blue to confirm equal loading. HIF-1α protein levels were quantified by measuring band intensities of HIF-1α, of which HeLa cells were exposed to SNAP (200 μM), spermine NONOate (200 μM), or hypoxia for 4 h. B, the mRNA level of VEGF (top) was detected by Northern analysis and the mRNA level of carbonic anhydrase 9 (bottom) by RT-PCR. By using quantitative real-time PCR, we measured mRNAs of VEGF and CA9, of which HeLa cells were exposed to SNAP (200 μM), spermine NONOate (200 μM), or hypoxia for 6 h. C, HRE activity was measured by transfecting HeLa cells with the HRE-driven luciferase reporter plasmid $p(HRE)_4$-luc (100 ng), together with a β-galactosidase encoding plasmid, pCHO110 (50 ng), into 1×10^5 HeLa cells. Luciferase activities were normalized by β-galactosidase activities. The values given are means and standard deviations of at least five experiments.

using molecular oxygen, α-ketoglutarate, vitamin C, and Fe(II). The hydroxylated prolines are recognized by the E3 ubiquitin ligase von Hippel-Lindau protein, after which HIF-1α is polyubiquitinated and degraded by the 26S proteasomal system (Semenza, 2000; Masson et al., 2001). To be a functional transactivator, the stabilized HIF-1α should be able to recruit its coactivator, CBP/p300. Under normoxic condition, HIF-1α is unable to interact with its coactivator. Asparagine 803 of HIF-1α is also hydroxylated under normoxic conditions by an oxygen-dependent asparagine hydroxylase, referred to as factor-inhibiting HIF-1 (FIH-1). The hydroxylated asparagine residue hinders the recruitment of CBP/p300, thereby inhibiting transactivation by the stabilized HIF-1α. A lack of oxygen reduces the activities of these two oxygen-dependent hydroxylases, so stabilizing the transactive form of HIF-1α (Hewitson et al., 2002; Lando et al., 2002).

The potential mechanisms by which NO donors activate the function of HIF-1α are as diverse as the NO donors are various (Brune and Zhou, 2007). Like other growth factors (Karni et al., 2002; Lauzier et al., 2007), the NO donor, 2,2′-(hydroxynitrosohydrazino) bis-ethanamine (NOC18), stimulates the translation of HIF-1α by activating the phosphatidylinositol-3 kinase and Akt pathways (Kasuno et al., 2004). With respect to the effect of endogenous NO, in mild hypoxia (5% oxygen), the resulting low NO concentrations (<400 nM) destabilize HIF-1α by inhibiting mitochondrial respiration, thereby increasing the local oxygen concentration in the cytosol, in which PHD2 is mainly located (Hagen et al., 2003; Mateo et al., 2003; Palacios-Callender et al., 2004). In contrast, high NO concentrations (>1 μM) stabilize HIF-1α by a nonmitochondrial pathway in both high- and low-oxygen concentrations (Mateo et al., 2003). Biotin switch assays revealed *S*-nitrosylation of the transactivation domain (amino acids 727–826) (Yasinska and Sumbayev, 2003; Cho et al., 2007) and the ODD domain of HIF-1α (Sumbayev et al., 2003; Li et al., 2007). It has been suggested that *S*-nitrosoglutathione (GSNO) inhibits PHD2-dependent VHL recruitment of HIF-1α in normoxic conditions (Metzen et al., 2003; Berchner-Pfannschmidt et al., 2007).

In the current study, we dissected the mechanism of activation of HIF-1α and tested the effects of the NO donor (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP) on each step. We found that SNAP did not block proline hydroxylation of HIF-1α by PHD2 but inhibited the interaction between VHL and hydroxylated HIF-1α, leading to the accumulation of HIF-1α. In contrast, SNAP directly inhibited the asparagine hydroxylation activity of FIH-1, so rescuing the interaction between HIF-1α and CBP.

Materials and Methods

Cells and Reagents. Human epithelial HeLa cells were cultured and exposed to hypoxia (1% O_2) as described previously (Choi et al., 2005). NO donors, *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate), NOC18, and SNAP, and zinc chelator *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) were purchased from Calbiochem (San Diego, CA), and all other chemicals were from Sigma Chemical (St. Louis, MO). The cDNAs used were HIF-1α (GenBank accession number U22431), FIH-1 (GenBank accession number AF395830), PHD2 (GenBank accession number AJ310543), and VHL (GenBank accession number AF010238) (Choi et al., 2005, 2006b).

Measurement of PHD Activity by VHL Pull-Down Assay. The catalytic domain (amino acids 184–418) of human PHD2 was cloned into the pET21b His2(+) vector, overexpressed in *Escherichia coli* as a histidine-tagged fusion protein, and purified by nickel-affinity chromatography (Choi et al., 2005). The in vitro VHL pull-down assay was performed as described previously (Jaakkola et al., 2001; Choi et al., 2005). In brief, [^{35}S]methionine-labeled VHL protein was synthesized by in vitro translation using the pcDNA3.1/hygro-VHL plasmid, according to the instruction manual (Promega, Madison, WI). GST-

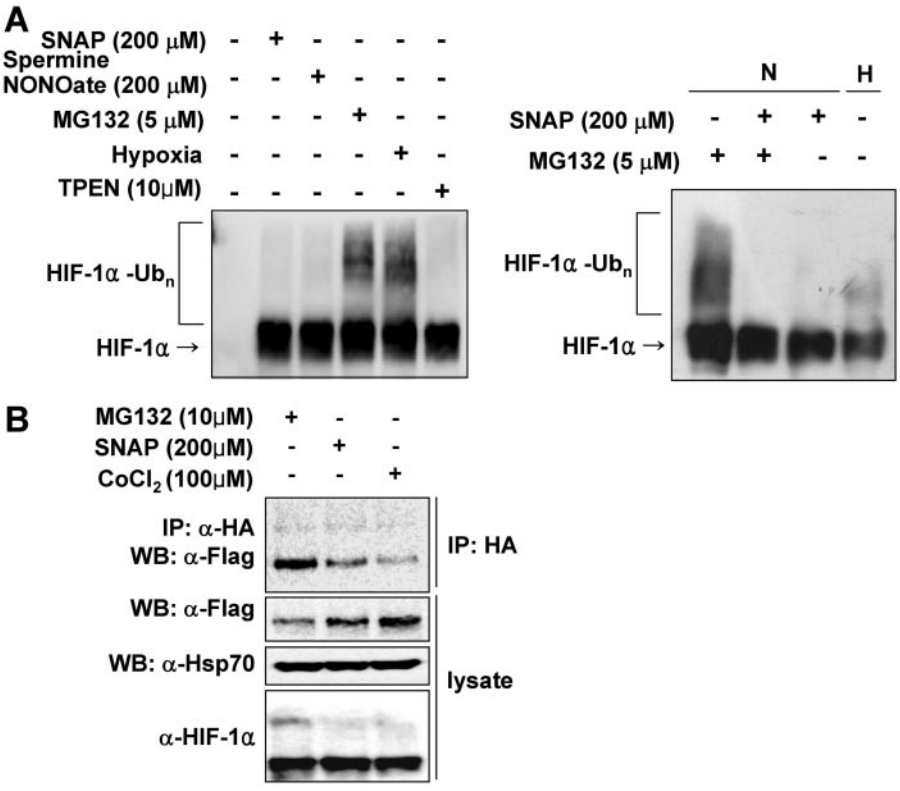

Fig. 2. Effect of SNAP on the ubiquitination of HIF-1α. A, HeLa cells were exposed to SNAP, spermine NONOate, the proteasome inhibitor MG132, hypoxia, and HIF-1α-ubiquitination blocker TPEN (Choi et al., 2006a) for 4 h under normoxic conditions. HIF-1α protein was detected by Western blot analysis. HIF-1α and ubiquitinated HIF-1α are indicated. N, normoxia; H, hypoxia (1% oxygen). B, 293 cells were transfected with Flag-HIF-1α and HA-ubiquitin as indicated. The transfected cells were treated with SNAP, MG132, or hypoxic mimicker $CoCl_2$ for 4 h before harvest. Whole cell lysates (300 μg) were immunoprecipitated (IP) with anti-HA antibody and then probed using anti-Flag antibody by Western blot (WB) analysis. HSP70 was detected to confirm equal loading.

ODD (amino acids 401–603 of human HIF-1α) was expressed in *E. coli* and purified with glutathione-resin (BD Biosciences). Resin-bound GST-ODD (2 μg of protein/~50 μl of resin volume) was incubated in the presence of 5 mM α-ketoglutarate with 1 to 2 μg of histidine-tagged PHD2 (184–418 amino acid) and other cofactors as indicated, in 200 μl of NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, and 1 mM phenylmethylsulfonyl fluoride) with mild agitation for 90 min at 30 °C. The reaction mixture was centrifuged and washed three times with 10 volumes of NETN buffer. Resin-bound GST-ODD was mixed with 10 μl of [^{35}S]-VHL in 500 μl of EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, and 0.25% Nonidet P40). After mild agitation at 4 °C for 2 h, the resin was washed three times with 1 ml of NETN buffer, and proteins were eluted in 3× SDS sample buffer, fractionated by 12% SDS-PAGE, and detected by autoradiography. The amount of each sample loaded was monitored by staining the GST-ODD with Coomassie blue. Instead of GST-ODD, HIF-1α peptide was used as substrate. Biotinylated human HIF-1α (amino acids 556–575) peptide (mol. wt. 2637.0) (biotin-DLDLEMLAPYIPMDDDFQLR; 2 μg) was preincubated with 1 μg of his-PHD2 (amino acids 184–418) in a final volume of 100 μl in NETN buffer containing 5 mM α-ketoglutarate with other cofactors as indicated at 30°C for 90 min. ImmunoPure immobilized monomeric avidin (Pierce Chemical) (30 μl of a 50% slurry) was preincubated with 3 mg of bovine serum albumin for 5 min at room temperature. The avidin was added to the above-mentioned hydroxylation reaction mixture, which was incubated with mild agitation for 60 min at 22°C. The avidin-associated peptide was washed three times with 1 ml of NETN buffer and then mixed with 10 μl of ^{35}S-labeled VHL in 500 μl of EBC buffer with mild agitation at 4°C for 1 h (Choi et al., 2005). The resin was washed four times with 1 ml of NETN buffer, and proteins were eluted, analyzed by 12% SDS-PAGE, and autoradiographed.

Measurement of FIH-1 Activity. Full-length human FIH-1 (amino acids 1–349) was cloned into pET28a vector (Novagen, Madison, WI), and FIH-1 was overexpressed in *E. coli* as a histidine-tagged fusion protein and purified by nickel-affinity chromatography. FIH-1 activity was measured by asparagine hydroxylation of FITC-HIF-1α peptide (amino acids 788–822) (mol. wt. 4332.2) (fluorescein-5-isothiocyanate-*aca*-DESGLPQLTSYDCEVNAPIQGSRNLLQGEELLRAL) or FITC-HIF-1α peptide (amino acids 786–826) C800A (fluorescein-5-isothiocyanate-*aca*-SMDESGLPQLTSYDAEVNAPIQGS RNLLQGEELLRALDQVN) (mol. wt. 4975.3) (AnyGen, GwangJu, Korea) developed for another assay (Cho et al., 2005, 2007). The peptide was incubated at a final concentration of 4 μM with 0.7 μg of recombinant FIH-1 with 100 μM α-ketoglutarate, 400 μM, or 2 μM vitamin C or and other cofactors as indicated, in a total volume of 50 μl for 2 h at room temperature (Cho et al., 2007).

Mass Spectrometric Analysis. After hydroxylation for 2 h at room temperature, excess salts were removed from substrate peptides with ZipTip$_{C18}$ (Millipore, Billerica, MA). The peptide was eluted from the tip with α-cyano-4-hydroxycinnamic acid in acetonitrile/water containing 0.1% trifluoroacetic acid [50:50 (v/v)] followed by extensive washing with 0.1% trifluoroacetic acid in water. The eluted peptide solution was transferred to a MALDI sample plate, and MALDI-TOF measurements were performed with a Voyager analyzer (Applied Biosystems, Foster City, CA).

Western Blot Analysis and Coimmunoprecipitation. HeLa cells were grown to 80% confluence on 100-mm tissue culture plates and treated with drugs or hypoxia for 4 h. Whole cell extracts were prepared as described previously (Choi et al., 2006b). For immunoprecipitation, 200 μg of whole cell lysates was incubated with 1 μg of anti-CBP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The resulting immunocomplexes were analyzed by Western blotting with anti-human HIF-1α antibody (BD Biosciences, San Jose, CA) (Choi et al., 2006b).

Transient Transfection and Luciferase Assay. Transfection of HRE-driven reporter plasmids (100 ng) and pCHO110 (50 ng), which encodes the β-galactosidase gene, into 1 × 10^5 HeLa cells was carried out using Lipofectamine Plus reagent (Choi et al., 2006b). Because the transfected β-galactosidase gene is transcribed by a constitutive promoter, β-galactosidase activity can represent the transfection efficiency of each sample. The measurements of luciferase activity were normalized for β-galactosidase.

Northern Analysis and Reverse Transcriptase PCR Analysis. Total RNA (10 μg) was used for Northern blot analysis. Blots were hybridized with 25 ng of α-^{32}P-labeled DNA fragments encoding vascular endothelial growth factor (VEGF) as described previously (Yim et al., 2003). Reverse-transcription PCR (RT-PCR) was performed with total RNA (1 μg), and 1 μM concentration of each primer of carbonic anhydrase-9 (CA9) (sense, 5′-CTGTCACTGCTGCTTCTGAT-3′; antisense, 5′-TCCTCTCCAGGTAGATCCTC-3′) (Choi et al., 2006b). The PCR products were analyzed on ethidium bromide-stained agarose gels.

Quantitative Real-Time RT-PCR. cDNA was quantified by real-time PCR on the iQTM SYBR Green Supermix and MyiQ single color real-time PCR detection system (Bio-Rad, Hercules, CA) were used. We used the following primers: human VEGF: forward, 5′-AACCATGAACTTTCTGCTGTCTTG-3′; reverse, 5′-TTCACCACTT-

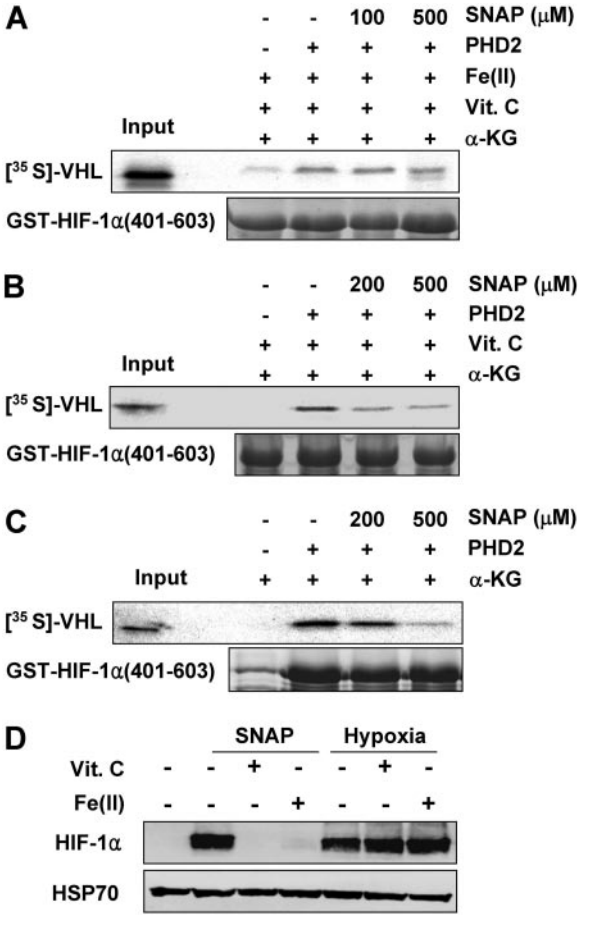

Fig. 3. Effects of SNAP on HIF-1α-proline hydroxylation-dependent VHL recruitment. Resin-bound GST-HIF-1α (amino acids 401–603) was incubated with recombinant His-tagged PHD2 (amino acids 184–418) (1 μg) in the presence or absence of SNAP with 100 μM Fe(II), 2 mM vitamin C, and 5 mM α-KG (A), with 2 mM vitamin C and 5 mM α-KG (B); or with only 5 mM α-KG (C). After the hydroxylation reaction, resin-bound GST-HIF-1α (amino acids 401–603) was washed and mixed with in vitro-translated ^{35}S-labeled VHL. [^{35}S]VHL capture by GST-HIF-1α (amino acids 401–603) was visualized by SDS-PAGE and autoradiography. Sample loading was monitored by measuring GST-HIF-1α (amino acids 401–603) protein stained with Coomassie blue. The first lane represents 10% of the [^{35}S]VHL used. D, HeLa cells were exposed to either SNAP (200 μM) or hypoxia (1% O_2) with or without vitamin C (100 μM) or $FeCl_2$ (100 μM) for 4 h. HIF-1α was detected by Western blot analysis. HSP70 was detected by Western blot analysis for loading control.

CGTGATGATTCTG-3′; human CA9: forward, 5′-CAGTTGCTGTC-TCGCTTGGA-3′; reverse, 5′-TGAAGTCAGAGGGCAGGAGTG-3′; and 18S, forward, 5′-ACCGCAGCTAGGAATAATGGAATA-3′; reverse, 5′-CTTTCGCTCTGGTCCGTCTT-3′. The expression level of 18S rRNA was used for normalization. All PCRs were performed in triplicate. We present the average and standard deviation of at least three experiments.

Results

NOC18-, Spermine NONOate-, and SNAP-Induced Stabilization of HIF-1α. To find NO-related drugs that are able to stabilize the HIF-1α protein in normoxic condition, we measured the protein level of HIF-1α in HeLa cells treated with several NO donors. The results in Fig. 1A showed that NOC18, spermine NONOate, and SNAP increase HIF-1α levels even in normoxic HeLa cells (Fig. 1, top). Based on these results, the following experiments focused on spermine NONOate and SNAP. We found that 2-h treatments with spermine NONOate or SNAP maximally increased the level of HIF-1α, and that 200 μM spermine NONOate and 100 μM SNAP maximally increased HIF-1α in normoxic HeLa cells to levels even greater than in hypoxic (1% O_2) cells. We then showed that the NO donors lead to the expression of target genes such as VEGF and CA9 after 2- to 3-h treatments (Fig. 1B). However, the levels of VEGF and CA9 were lower than those induced by hypoxia. Transient transfection analysis (Fig. 1C) also showed that spermine NONOate and SNAP activated the expression of HRE-driven luciferase genes.

SNAP Blocks VHL Binding of HIF-1α. To test whether SNAP acts by blocking ubiquitination, we examined the effect of the protease inhibitor MG132. Western blot analysis showed that treatment with MG132 stabilized the ubiquitinated high molecular weight form of HIF-1α in normoxic conditions. Zinc chelator TPEN specifically inhibits the ubiquitination of HIF-1α (Choi et al., 2006a). Like TPEN, SNAP and spermine NONOate reduce the high molecular weighted HIF-1α and indeed inhibit ubiquitination (Fig. 2). We co-transfected HA-tagged ubiquitin and Flag-tagged HIF-1α and then performed coimmunoprecipitation assay by using anti-HA antibody. We confirmed that SNAP reduced the ubiquitinated form of HIF-1α (Fig. 2B). O_2-dependent ubiquitination of HIF-1α is mediated by an HIF-1α-specific E3 ligase named VHL. Because VHL specifically recognizes and binds to the hydroxylated proline residues of the ODD domain (amino acids 401–603), HIF-1α-specific proline hydroxylation is the initial event in HIF-1α degradation. To assess the effect of SNAP on the hydroxylation and VHL binding ability of HIF-1α, we used bacterially expressed and

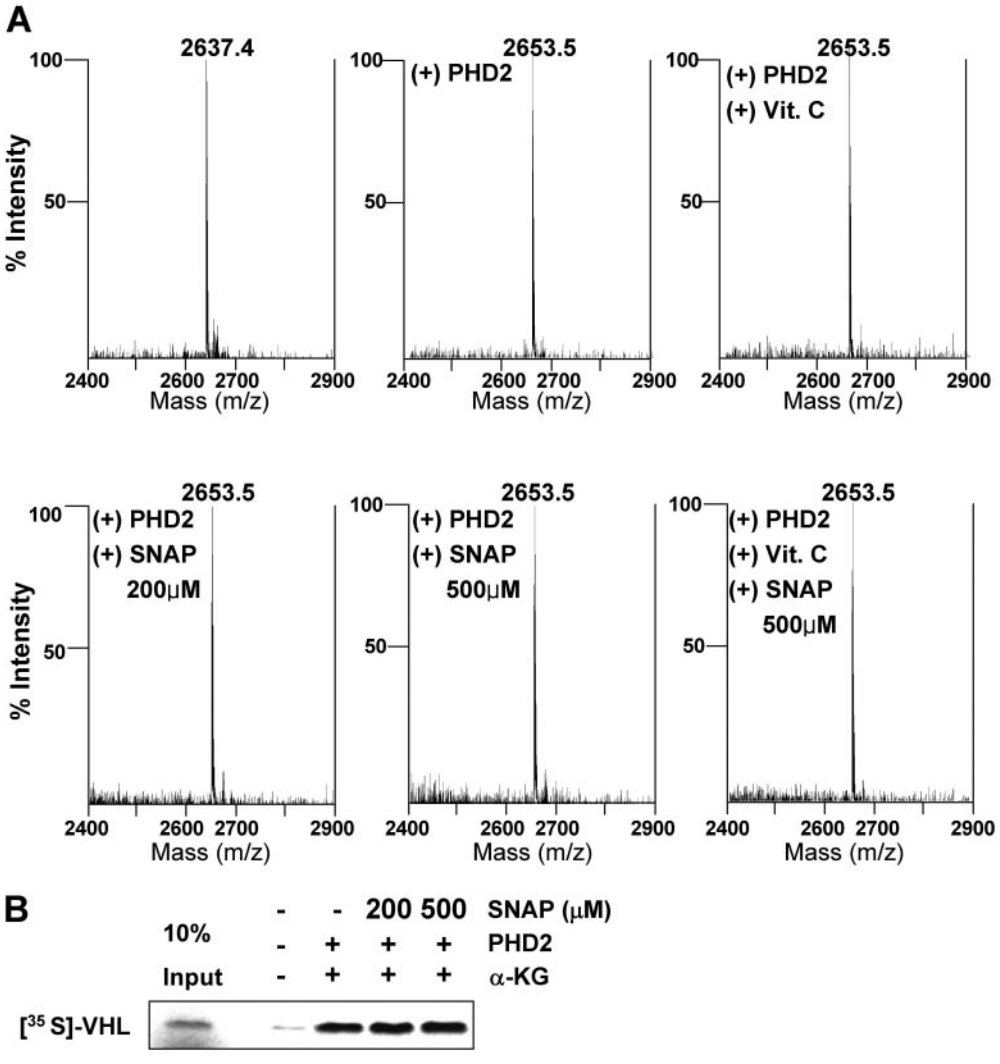

Fig. 4. Effect of SNAP on proline-hydroxylation of HIF-1α. A, 2 μM biotin-HIF-1α-(556–575) peptide (mol. wt. 2637.0) and 100 μM α-KG were incubated together with the following combinations of the three reagents: 1 μg of recombinant His-PHD2 (amino acids 184–418), 400 μM vitamin C, and SNAP as indicated; Fe(II) was not included. The reaction mixtures were analyzed by MALDI-TOF as described previously (Cho et al., 2007). The detected mass of the major peptide was shown above the peak. B, 2 μg of biotin-HIF-1α (amino acids 556–575) peptide (mol. wt. 2637.0) was hydroxylated in the absence or presence of SNAP by adding recombinant His-PHD2 (amino acids 184–418) (1 μg) and 400 μM α-KG. Biotin-HIF-1α (amino acids 556–575) peptide was then isolated using immobilized avidin-resin and mixed with labeled VHL (Choi et al., 2005). [^{35}S]VHL capture by hydroxylated biotin-HIF-1α (amino acids 556–575) peptide was visualized by SDS-PAGE and autoradiography. The first lane represents 10% of the [^{35}S]VHL used.

purified GST-HIF-1α (amino acids 401–603) fusion protein as substrate. Because we used the glutathione resin-bound form of GST-HIF-1α (amino acids 401–603), glutathione was not present in the subsequent reactions. GST-HIF-1α (amino acids 401–603) was incubated with the purified recombinant histidine-PHD2 (amino acids 184–418) fusion protein, which contains the catalytic domain, in the presence of α-ketoglutarate, Fe (II), and vitamin C (Choi et al., 2005). The addition of PHD2 increased capture of VHL protein, indicating that it hydroxylates the proline residues of HIF-1α (Fig. 3A). We added SNAP to the resin-bound GST-HIF-1α together with PHD2 and cofactors as indicated (Fig. 3A). The presence of SNAP (100 and 500 μM) in the hydroxylation reaction did not significantly inhibit the recruitment of VHL by HIF-1α.

Recent studies have suggested that NO donors modify the activities of target proteins by *S*-nitrosylation of their cysteine residues. *S*-Nitrosylation is a nonenzymatic oxidation reaction between the thiols of specific cysteine residues and reactive N_2O_3-like species (Ahern et al., 2002). Excess Fe(II) and vitamin C in the hydroxylation reaction can mask any oxidative effects of NO (Sumbayev et al., 2003; Li et al., 2007). The absence of both Fe(II) and vitamin C did not reduce the activity of PHD2 in vitro (Cho et al., 2005). The results in Fig. 3B reveal that in the absence of Fe(II), PHD2 still increased the hydroxylation-dependent VHL recruitment, indicating that it hydroxylates the proline residues of HIF-1α. It is interesting that, in this condition, SNAP became able to reduce the capture of VHL. In the absence of both Fe(II) and vitamin C, PHD2 still increased the capture of VHL, whereas SNAP inhibited VHL recruitment (Fig. 3C). These results indicate that SNAP only inhibits the hydroxylation-dependent binding of HIF-1α by VHL when reducing agents such as Fe(II) and vitamin C are limiting. To confirm these findings, we treated HeLa cells with vitamin C (100 μM) and SNAP (200 μM) for 4 h. The results in Fig. 3D show that both vitamin C and Fe(II) abolish the effect of SNAP on HIF-1α stability, whereas vitamin C and Fe(II) fail to reduce the HIF-1α protein, which was stabilized by hypoxia (Lu et al., 2005).

SNAP Fails to Inhibit Proline Hydroxylation. To determine whether SNAP inhibits the hydroxylation reaction or VHL recruitment, we directly assessed its effect on hydroxylation of Pro564 of HIF-1α by measuring changes in the molecular weight of HIF-1α peptide (amino acids 556–575) by mass spectroscopy. For the hydroxylation reaction we used purified His-tagged PHD2 (amino acids 184–418), and α-ketoglutarate, in the absence of Fe(II). After incubation with PHD2, the peptide yielded a new MALDI-TOF peak corresponding to an increase of molecular weight of 16, both in the presence and the absence of vitamin C (Fig. 4A). The molecular weight also increased in the presence of SNAP, indicating that SNAP stabilizes HIF-1α by inhibiting the interaction between VHL and hydroxylated HIF-1α rather than by inhibiting the proline-hydroxylation reaction. To confirm this, we used the HIF-1α peptide (amino acids 556–575) in the hydroxylation-dependent VHL pull-down assay instead of GST-HIF-1α (amino acids 401–603). The ODD domain of HIF-1α contains one cysteine residue (residue 520), but the HIF-1α peptide contains no cysteine. PHD2 increased the capture of VHL protein, indicating that it hydroxylates the peptide. However, SNAP failed to inhibit VHL recruitment by the HIF-1α peptide even in the absence of Fe(II) and vitamin C, suggesting that SNAP inhibition requires the cysteine residue of HIF-1α (Fig. 4B). As a control experiment, we added the purified GST protein in the HIF-1α peptide (556–575)/VHL pull-down assay. The results showed that GST alone had no effect on association of HIF-1α peptide (amino acids 556–575) with VHL in the presence of SNAP, confirming that the cysteine residue of HIF-1α-ODD but not of GST mediates the effect of SNAP (supplemental data).

Involvement of Cysteine 520 of HIF-1α. To test whether *S*-nitrosylation is involved, we created a point mutation substituting alanine for cysteine 520. The result shown in Fig. 5 indicates that replacement of the cysteine abolishes the response of HIF-1α to SNAP in the hydroxylation-dependent VHL pull-down assay, implying that *S*-nitrosylation of Cys520 blocks the interaction between hydroxylated HIF-1α and VHL. Our results indicate that SNAP stabilizes HIF-1α by blocking VHL recruitment not by inhibiting proline hydroxylation. The *S*-nitrosylation of Cys520 presumably prevents hydroxylated HIF-1α from recruiting VHL.

Effects of SNAP on Asparagine Hydroxylation and CBP Binding of HIF-1α. To induce transactivation capability, the stabilized HIF-1α needs to recruit a coactivator, CBP/p300. In normoxic conditions, the asparagine 803 is hydroxylated by FIH-1, and this interferes with the interaction between CBP/p300 and HIF-1α. Therefore, FIH-1 activity is inversely related to the recruitment of CBP/p300. To test the effect of SNAP on FIH-1 activity, we incubated the HIF-1α peptide (amino acids 788–822) with purified recombinant His-tagged FIH-1 in the presence and absence of SNAP. After treatment with FIH-1, α-ketoglutarate (α-KG), and a minimal amount of vitamin C (2 μM) in the absence of Fe(II), the peptide gave the characteristic new MALDI-TOF peak (Fig. 6A) (Cho et al., 2007). SNAP prevented FIH-1 from hydroxylating the peptide (Fig. 6A), whereas its effect was abolished in the presence of excess vitamin C (400 μM) (Fig.

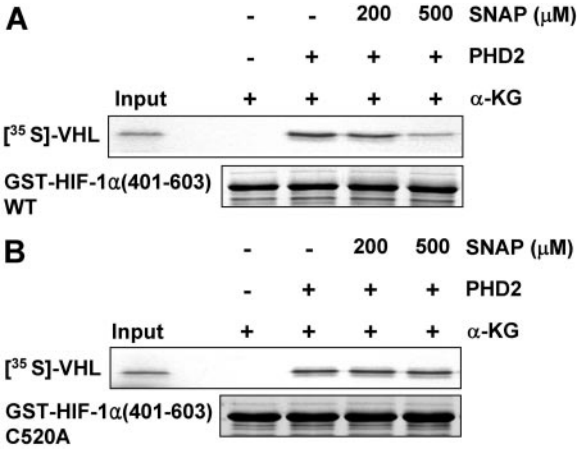

Fig. 5. Effect of SNAP and cysteine 520 of HIF-1α. A, resin-bound GST-HIF-1α (amino acids 401–603) was incubated with his-PHD2 (amino acids 184- 418) (1 μg) and 5 mM α-KG in the presence or absence SNAP (200 or 500 μM) for 90 min at 30°C. Then the resin-bound GST-HIF-1α (amino acids 401–603) was washed and mixed with in vitro translated ^{35}S-labeled VHL. [^{35}S]-VHL capture by GST-HIF-1α(401–603) was visualized by SDS-PAGE and autoradiography. Sample loading was monitored by measuring GST-HIF-1α (amino acids 401–603) protein stained with Coomassie blue. The first lane represents 10% of the [^{35}S]VHL used. B, resin-bound GST-HIF-1α (amino acids 401–603) C520A mutant protein was used instead of GST-HIF-1α (amino acids 401–603).

6B). Thus the asparagine-hydroxylating activity of FIH-1 is only inhibited by SNAP when reducing agents are limiting.

HIF-1α peptide (amino acids 786–826) contains one cysteine. To test whether the inhibitory effect of SNAP on FIH-1 activity depends on this residue, we used the alanine-substituted mutant peptide (amino acids 786–826). The mass analysis in Fig. 7A shows that FIH-1 was still able to increase the molecular weight of the mutant HIF-1α peptide and that SNAP also inhibited its hydroxylation. The fact that cysteine 800 is not essential for the inhibitory effect of SNAP on the asparagine hydroxylation of HIF-1α implies that SNAP does not inhibit FIH-1 activity by *S*-nitrosylation of this residue.

To confirm that SNAP increases the stability and the transactivation of HIF-1α by inhibiting both VHL recruitment and asparagine hydroxylation, we tested by coimmunoprecipitation whether HIF-1α stabilized by SNAP was able

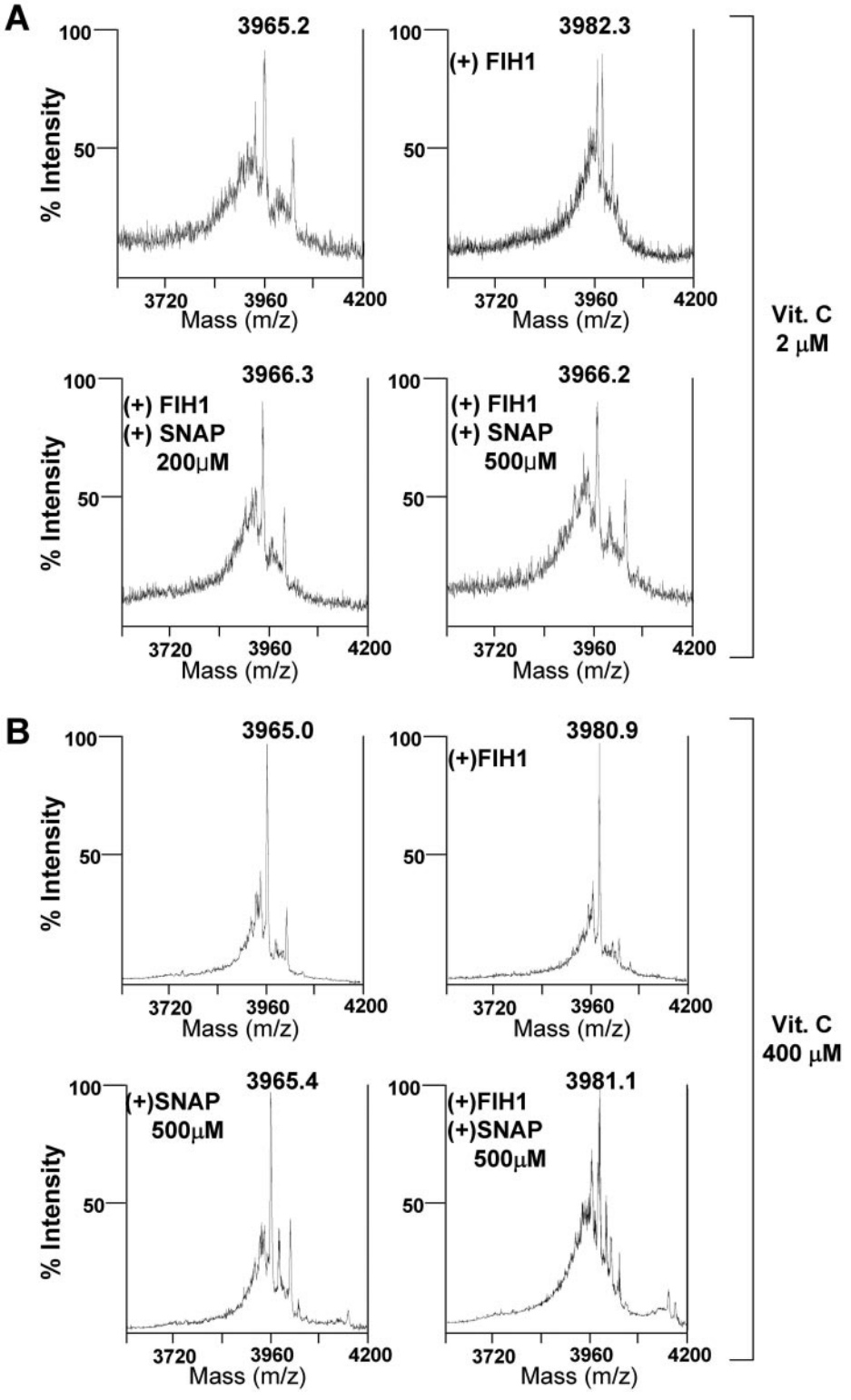

Fig. 6. Effects of NO donors on asparagine hydroxylation of HIF-1α. A, FITC-HIF-1α (amino acids 788–822) peptide (mol. wt. 4332.2) (3 μM), α-KG (100 μM), and vitamin C (2 μM) were mixed with His-tagged full-length FIH-1 (1 μg) in hydroxylation buffer in the presence or absence of SNAP (200 or 500 μM). B, FITC-HIF-1α (amino acids 788–822) peptide (3 μM), α-KG (100 μM), and vitamin C (400 μM) were mixed with His-FIH-1 (1 μg) in the presence or absence of SNAP (500 μM). Fe(II) was not added. The mixtures were incubated at 30°C for 2 h, followed by MALDI-TOF analysis. Note that the indicated molecular masses correspond to the peptides with detached FITC (mol wt. 389.0) in a sodium (mol. wt. 23.0)-added form (mol. wt. 3965.7) during the MALDI-TOF measurements. The detected mass of the major peptide is shown above the peak.

to interact with its coactivator, CBP. The results in Fig. 7B show that HIF-1α stabilized by SNAP or spermine NONOate was able to interact with CBP in the cellular context (Fig. 7B). These results suggest that SNAP has two distinct inhibitory activities: one blocks the interaction between proline-hydroxylated HIF-1α and VHL, thereby leading to the accumulation of HIF-1α; the other prevents FIH-1 from hydroxylating the asparagine residue of HIF-1α. In this way, SNAP keeps HIF-1α able to interact with CBP.

Discussion

We have confirmed that SNAP stabilizes the transactive form of HIF-1α, leading to the induction of HIF-1α target genes such as VEGF and CA9. We examined the direct effects of NO donors on the hydroxylation of HIF-1α peptides by PHD2 and FIH-1 in vitro. The finding that SNAP reduced the high molecular weight form of HIF-1α indicated that it inhibits the ubiquitination of HIF-1α, thereby stabilizing it. SNAP treatment failed to stabilize cellular HIF-1α when the cells were cotreated with vitamin C, indicating that cellular redox status affects SNAP action, presumably NO release from SNAP. Our observations imply that FIH-1, but not its substrate HIF-1α, is directly inhibited by SNAP. In agreement with this, the coactivator CBP was able to interact with HIF-1α stabilized by SNAP in the cellular context.

It has been reported that the NO donors GSNO and SNAP inhibit the proline hydroxylation-dependent VHL recruitment (Metzen et al., 2003). In contrast, others found that NO donors, including SNP, PAPA NONOate, and MAHMA NONOate, rather increase the proline hydroxylation-dependent VHL interaction (Wang et al., 2002). However, Li et al. (2007) demonstrated recently, using an antibody directed against the hydroxylated proline of HIF-1α, that in GSNO-treated mouse 4T tumor cells, proline-hydroxylated HIF-1α remained detectible. They also demonstrated by a biotin switch assay, that Cys533 of mouse HIF-1α (equivalent to Cys520 of human HIF-1α) is nitrosylated, and that this *S*-nitrosylation does not inhibit proline hydroxylation. By using hydroxylation-dependent VHL pull-

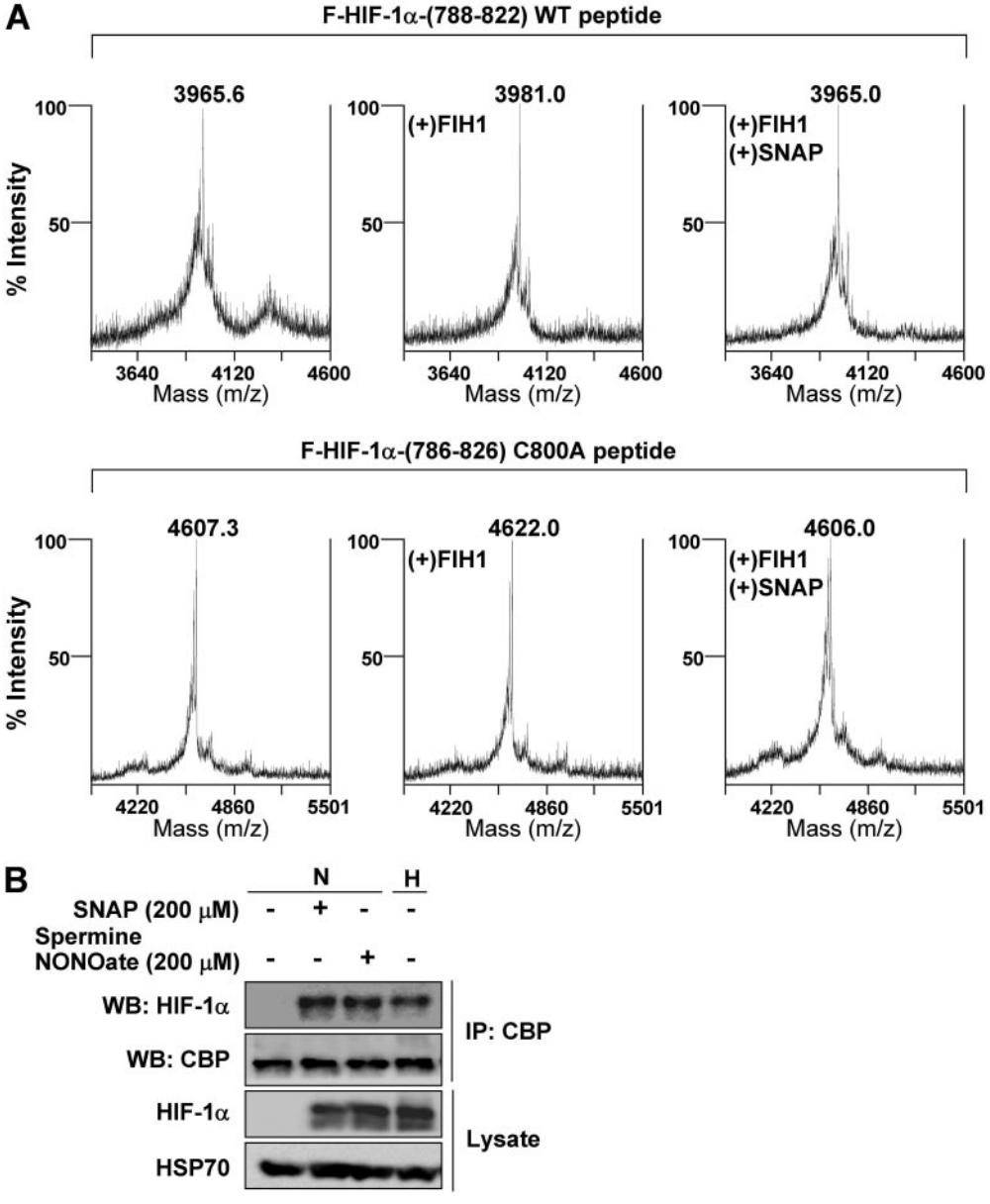

Fig. 7. The effect of SNAP and cysteine 800 of HIF-1α. A, in the presence or absence of SNAP (500 μM), 3 μM FITC-HIF-1α (amino acids 788–822) peptide (top) or 3 μM FITC-HIF-1α (amino acids 786–826) C800A peptide (mol. wt. 4975.3) (bottom) was incubated along with His-FIH-1 (1 μg) with α-KG (100 μM) and vitamin C (2 μM) at 30°C for 2 h followed by MALDI-TOF analysis (Cho et al., 2007). Note that the indicated molecular weights correspond to the peptides with detached FITC (mol. wt. 389.0) in a sodium (mol. wt. 23.0)-added form (mol. wt. 4608.8) during the MALDI-TOF measurements. The detected mass of the major peptide is shown above the peak. B, effects of SNAP and spermine NONOate on CBP recruitment by HIF-1α in vivo. HeLa cells were exposed to SNAP (200 μM), spermine NONOate (200 μM), or hypoxic conditions (1% oxygen) for 4 h. Whole cell lysates were immunoprecipitated with anti-CBP antibody, and the resulting immunocomplexes were analyzed using anti-human HIF-1α and anti-CBP. To confirm equal loading, HSP70 was detected with anti-HSP70 antibody.

down assay and mass analysis, we confirmed here that SNAP blocks VHL recruitment but not proline hydroxylation of HIF-1α, and that this inhibitory effect is reversed by reducing agents such as vitamin C and Fe(II).

To generate the transactive form of HIF-1α, activators need to repress the dual control system consisting of the PHD2/VHL and FIH-1/CBP pathways (Fig. 8). Here, we first showed that asparagine hydroxylation by FIH-1 is inhibited by SNAP, thereby maintaining the affinity of HIF-1α for CBP. Because the nitrosylation reaction involves chemical chain reactions, the preservation and detection of nitrosylated proteins needs very sophisticated methods, both in vivo and in vitro. By separating HIF-1α (amino acids 786–826) peptide using reverse-phase high-performance liquid chromatography instead of mass analysis, we confirmed that the purified HIF-1α (amino acids 786–826) peptide can be *S*-nitrosylated at cysteine 800 by an excess SNAP (2 mM) in vitro (Cho et al., 2007). However, MALDI-TOF analysis failed to detect any *S*-nitrosylated HIF-1α (Fig. 6) presumably because the *S*-NO bond of the *S*-nitrosopeptide is labile and readily disrupted in the gas phase of a mass spectrometer by in-source decay (Kaneko and Wada, 2003). Because NO increases not only the stability but also the transactivation of HIF-1α, *S*-nitrosylation of Cys800 is expected to increase its interaction with the coactivator CBP/p300 (Yasinska and Sumbayev, 2003; Sumbayev and Yasinska, 2007). Using a fluorescence polarization-based interaction assay (Cho et al., 2005), we found that *S*-nitrosylation of HIF-1α (amino acids 786–826) peptide instead decreased the p300 interaction. In addition, SNAP inhibited the hydroxylation of Asn803 of HIF-1α peptide, which does not require the presence of cysteine 800 (Fig. 7). These results suggested that NO increases p300/CBP recruitment not by Cys800 nitrosylation of HIF-1α but by inhibiting FIH-1. We assume that SNAP inhibits FIH-1 by either oxidation of the Fe(II) in its catalytic core or by nitrosylation of some cysteine residues (Hewitson et al., 2007).

The importance of HIF-1α has been emphasized in the context of therapeutic interventions in many diseases, especially cancer and recently inflammation. The finding that the catalytic activities of both PHDs and FIH-1 require vitamin C, Fe(II), α-ketoglutarate, and molecular oxygen provides insight into how HIF-1α can be activated by changes in mitochondrial activity, ROS or the pool of reducing agents including NADH, vitamin C, and glutathione. The inhibitory effect of antioxidants including vitamin C (Lu et al., 2005; Gao et al., 2007) and Fe(II) (Gerald et al., 2004; Lu et al., 2005) on tumorigenesis has been re-evaluated in the context of HIF-1α destabilization. Gao et al. (2007) reported that antioxidants such as *N*-acetylcysteine and vitamin C inhibit lymphoma xenografts by diminishing HIF-1 levels in a prolyl hydroxylase 2- and von Hippel-Lindau protein-dependent manner (Gao et al., 2007). In addition, trichloroacetic acid cycle intermediates are involved in the HIF-1α hydroxylation reaction. For PHDs, succinate as a product is a competitive inhibitor of α-ketoglutarate, the substrate (Selak et al., 2005; Pan et al., 2007). Fumarate and malate are other trichloroacetic acid cycle intermediates shown to negatively regulate PHD (Isaacs et al., 2005; Pan et al., 2007) but not FIH activity (Hewitson et al., 2007; Koivunen et al., 2007). In addition, mitochondrial ROS are essential for proper O_2 sensing by PHDs. In cytochrome *c* null embryonic cells, which have defects in generating mitochondrial ROS, HIF-α protein remains hydroxylated by PHDs and so cannot be stabilized even in moderate hypoxia (1.5% O_2 for 4 h) (Mansfield et al., 2005), whereas exogenous treatment with H_2O_2 restores HIF-α stabilization even in the absence of cytochrome *c*.

Although PHDs and FIH-1 share common cofactors, the catalytic domains of both enzymes are different. FIH-1 has jumonji domain, which differs from the catalytic domain of PHD2. These two hydroxylation enzymes respond differently to inhibitors. Succinate and fumarate inhibit PHD but not FIH-1 activity (Hewitson et al., 2007; Koivunen et al., 2007). Desferrioxamine and several metals are effective inhibitors of FIH-1 but ineffective inhibitors of PHDs in vitro (Hirsilä et al., 2005). Here, we demonstrate that SNAP inhibits FIH-1 but not PHD2. Instead, it inhibits VHL recruitment, stabilizing the transactive form of HIF-1α in normoxic cells. NO is released in many pathophysiological conditions, including inflammation and blood clotting (Vadseth et al., 2004; Li et al., 2007; Yeo et al., 2008). In these conditions, a variety of mediators may interfere with the NO effects on the regulation of HIF-1α. It remains to be seen whether the endogenous NO also has dual inhibitory effects on VHL recruitment and asparagine hydroxylation, leading to functional HIF-1α, and whether the NO effects can be modulated by other inflammatory mediators (Li et al., 2007).

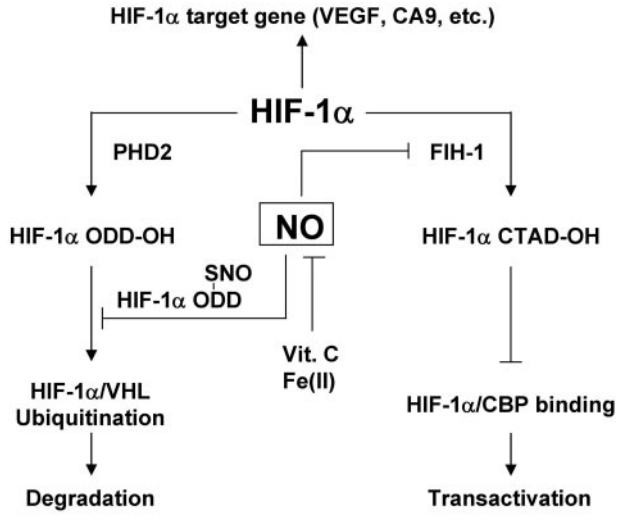

Fig. 8. Schematic diagram of the effects of SNAP on the HIF-1α regulation. For details, see *Discussion*.

References

Ahern GP, Klyachko VA, and Jackson MB (2002) cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. *Trends Neurosci* **25:**510–517.

Berchner-Pfannschmidt U, Yamac H, Trinidad B, and Fandrey J (2007) Nitric oxide modulates oxygen sensing by hypoxia-inducible factor 1-dependent induction of prolyl hydroxylase 2. *J Biol Chem* **282:**1788–1796.

Brune B and Zhou J (2007) Nitric oxide and superoxide: interference with hypoxic signaling. *Cardiovasc Res* **75:**275–282.

Cho H, Ahn DR, Park H, and Yang EG (2007) Modulation of p300 binding by posttranslational modifications of the C-terminal activation domain of hypoxia-inducible factor-1alpha. *FEBS Lett* **581:**1542–1548.

Cho H, Park H, and Yang EG (2005) A fluorescence polarization-based interaction assay for hypoxia-inducible factor prolyl hydroxylases. *Biochem Biophys Res Commun* **337:**275–280.

Choi KO, Lee T, Lee N, Kim JH, Yang EG, Yoon JM, Kim JH, Lee TG, and Park H (2005) Inhibition of the catalytic activity of hypoxia-inducible factor-1α-prolyl-hydroxylase 2 by a MYND-type zinc finger. *Mol Pharmacol* **68:**1803–1809.

Choi SM, Choi KO, Lee N, Oh M, and Park H (2006a) The zinc chelator, *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine, increases the level of nonfunctional HIF-1alpha protein in normoxic cells. *Biochem Biophys Res Commun* **343:**1002–1008.

Choi SM, Choi KO, Park YK, Cho H, Yang EG, and Park H (2006b) Clioquinol, a Cu(II)/Zn(II) chelator, inhibits both ubiquitination and asparagine hydroxylation of hypoxia-inducible factor-1α, leading to expression of vascular endothelial growth factor and erythropoietin in normoxic cells. *J Biol Chem* **281:**34056–34063.
Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, Bhujwalla ZM, Felsher DW, Cheng L, Pevsner J, et al. (2007) HIF-dependent antitumorigenic effect of antioxidants in vivo. *Cancer Cell* **12:**230–238.
Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ, Mansuy D, Pouysségur J, Yaniv M, and Mechta-Grigoriou F (2004) JunD reduces tumor angiogenesis by protecting cells from oxidative stress. *Cell* **118:**781–794.
Hagen T, Taylor CT, Lam F, and Moncada S (2003) Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. *Science* **302:**1975–1978.
Hewitson KS, Lienard BM, McDonough MA, Clifton IJ, Butler D, Soares AS, Oldham NJ, McNeill LA, and Schofield CJ (2007) Structural and mechanistic studies on the inhibition of the hypoxia-inducible transcription factor hydroxylases by tricarboxylic acid cycle intermediates. *J Biol Chem* **282:**3293–3301.
Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, et al. (2002) Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J Biol Chem* **277:**26351–26355.
Hirsilä M, Koivunen P, Xu L, Seeley T, Kivirikko KI, and Myllyharju J (2005) Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. *FASEB J* **19:**1308–1310.
Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, Chung YL, Merino M, Trepel J, Zbar B, Toro J, et al. (2005) HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell* **8:**143–153.
Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, et al. (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* **292:**468–472.
Kaneko R and Wada Y (2003) Decomposition of protein nitrosothiolsin matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry. *J Mass Spectrom* **38:**526–530.
Karni R, Dor Y, Keshet E, Meyuhas O, and Levitzki A (2002) Activated pp60c-Src leads to elevated hypoxia-inducible factor (HIF)-1alpha expression under normoxia. *J Biol Chem* **277:**42919–42925.
Kasuno K, Takabuchi S, Fukuda K, Kizaka-Kondoh S, Yodoi J, Adachi T, Semenza GL, and Hirota K (2004) Nitric oxide induces hypoxia-inducible factor 1 activation that is dependent on MAPK and phosphatidylinositol 3-kinase signaling. *J Biol Chem* **279:**2550–2558.
Koivunen P, Hirsilä M, Remes AM, Hassinen IE, Kivirikko KI, and Myllyharju J (2007) Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF. *J Biol Chem* **282:**4524–4532.
Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, and Bruick RK (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* **16:**1466–1471.
Lauzier MC, Pagé EL, Michaud MD, and Richard DE (2007) Differential regulation of hypoxia-inducible factor-1 through receptor tyrosine kinase transactivation in vascular smooth muscle cells. *Endocrinology* **148:**4023–4031.
Li F, Sonveaux P, Rabbani ZN, Liu S, Yan B, Huang Q, Vujaskovic Z, Dewhirst MW, and Li CY (2007) Regulation of HIF-1alpha stability through S-nitrosylation. *Mol Cell* **26:**63–74.
Lu H, Dalgard CL, Mohyeldin A, McFate T, Tait AS, and Verma A (2005) Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. *J Biol Chem* **280:**41928–41939.
Mansfield KD, Guzy RD, Pan Y, Young RM, Cash TP, Schumacker PT, and Simon MC (2005) Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation. *Cell Metab* **1:**393–399.
Masson N, Willam C, Maxwell PH, Pugh CW, and Ratcliffe PJ (2001) Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J* **20:**5197–5206.
Mateo J, Garcia-Lecea M, Cadenas S, Hernández C, and Moncada S (2003) Regulation of hypoxia-inducible factor-1alpha by nitric oxide through mitochondria-dependent and -independent pathways. *Biochem J* **376:**537–544.
Metzen E, Zhou J, Jelkmann W, Fandrey J, and Brüne B (2003) Nitric oxide impairs normoxic degradation of HIF-1alpha by inhibition of prolyl hydroxylases. *Mol Biol Cell* **14:**3470–3481.
Palacios-Callender M, Quintero M, Hollis VS, Springett RJ, and Moncada S (2004) Endogenous NO regulates superoxide production at low oxygen concentrations by modifying the redox state of cytochrome c oxidase. *Proc Natl Acad Sci U S A* **101:**7630–7635.
Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ, and Simon MC (2007) Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. *Mol Cell Biol* **27:**912–925.
Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, and Gottlieb E (2005) Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell* **7:**77–85.
Semenza GL (2000) HIF-1 and human disease: one highly involved factor. *Genes Dev* **14:**1983–1991.
Sumbayev VV, Budde A, Zhou J, and Brüne B (2003) HIF-1 alpha protein as a target for S-nitrosation. *FEBS Lett* **535:**106–112.
Sumbayev VV and Yasinska IM (2007) Mechanisms of hypoxic signal transduction regulated by reactive nitrogen species. *Scand J Immunol* **65:**399–406.
Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, Torbet J, Vilaire G, Bennett JS, Murciano JC, et al. (2004) Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. *J Biol Chem* **279:**8820–8826.
Wang F, Sekine H, Kikuchi Y, Takasaki C, Miura C, Heiwa O, Shuin T, Fujii-Kuriyama Y, and Sogawa K (2002) HIF-1alpha-prolyl hydroxylase: molecular target of nitric oxide in the hypoxic signal transduction pathway. *Biochem Biophys Res Commun* **295:**657–662.
Yasinska IM and Sumbayev VV (2003) S-nitrosation of Cys-800 of HIF-1alpha protein activates its interaction with p300 and stimulates its transcriptional activity. *FEBS Lett* **549:**105–109.
Yeo WS, Lee SJ, Lee JR, and Kim KP (2008) Nitrosative protein tyrosine modifications: biochemistry and functional significance. *BMB Rep* **41:**194–203.
Yim S, Choi SM, Choi Y, Lee N, Chung J, and Park H. (2003) Insulin and hypoxia share common target genes but not the hypoxia-inducible factor-1α. *J Biol Chem* **278:**38260–38268.

Address correspondence to: Dr. Hyunsung Park, 90 Cheonnong-dong, Tongdaemun-gu, Seoul, 130-743, Republic of Korea. E-mail: hspark@uos.ac.kr