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THE NITRIC OXIDE DONOR, SNAP, STABILIZES TRANS-ACTIVE HYPOXIA-INDUCIBLE FACTOR-1α BY INHIBITING VHL RECRUITMENT AND ASPARAGINE HYDROXYLATION

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Running Title: SNAP-induced trans-active HIF-1α

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Abbreviations used are: α-KG, α-ketoglutarate; CA9, carbonic anhydrase 9; CBP, cAMP responsive element binding protein; FIH-1, factor inhibiting HIF-1α; HIF-1α, hypoxia-inducible factor-1α; HRE, hypoxia-responsive element; ODD, oxygen depenent degradation domain of HIF-1α ;PHD, HIF-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.

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ABSTRACTS

We have confirmed that the NO donor, SNAP, stabilizes the *trans*-active form of Hypoxia-Inducible Factor-1α (HIF-1α), leading to the induction of HIF-1α target genes such as VEGF and CA9. 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, CBP/p300. We examined the effects of NO donor, SNAP on proline and asparagines 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 asparagines 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.

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INTRODUCTION

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 posttranslational 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) using molecular oxygen, α-ketoglutarate, vitamin C, and Fe(II) . The hydroxylated prolines are recognized by the E3 ubiquitin ligase, von Hippel-Lindau protein (pVHL), following which HIF-1α is polyubiquitinated and degraded by the 26S proteasomal system (Masson et al., 2001; Semenza, 2000). 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, NOC18, stimulates translation of HIF-1α by activating the PI3 kinase and Akt pathways (Kasuno et al., 2004). With respect to the effect of endogenous NO, in mild hypoxia (5%

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oxygen), the resulting low NO concentrations (< 400 nM) destabilize HIF-1α by inhibiting mitochondrial respiration, thereby increasing the local oxygen concentration in the cytosol where 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 non-mitochondrial pathway in both high and low oxygen concentrations (Mateo et al., 2003). Biotin switch assays revealed S-nitrosylation of the transactivation domain (727-826 amino acids) (Cho et al., 2007; Yasinska and Sumbayev, 2003) and the ODD domain of HIF-1α (Li et al., 2007; Sumbayev et al., 2003). It has been suggested that S-nitrosoglutathione (GSNO) inhibits PHD2-dependent VHL recruitment of HIF-1α in normoxic conditions (Berchner-Pfannschmidt et al., 2007; Metzen et al., 2003).

In the current study we dissected the mechanism of activation of HIF-1α and tested the effects of the NO donor 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 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.

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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, spermine NONOate (N-(2-Aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine), NOC18 (2,2'-(hydroxynitrosohydrazino) bis-ethanamine), and SNAP ((±)-S-nitroso-N-acetylpenicillamine); zinc chelator, TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine) were purchased from Calbiochem, and all other chemicals from Sigma Chemical. The cDNAs used were HIF-1α (U22431), FIH-1(AF395830), PHD2 (AJ310543), and VHL (AF010238) (Choi et al., 2005; Choi et al., 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 (Choi et al., 2005; Jaakkola et al., 2001). Briefly, [^{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). GST-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 1-2 μg of histidine-tagged PHD2 (184-418 amino acid) and other cofactors as indicated, in 200 μl of NETN buffer, 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. 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 3x SDS sample buffer, fractionated by 12% SDS-PAGE, and

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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 can be used as substrate. Biotinylated human HIF-1α (556 to 575 amino acids) peptide (MW 2637.0) (Biotin-DLDLEMLAPYIPMDDDFQLR) (2 μg) was preincubated with 1 μg of his-PHD2 (184-418 amino acid) 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 (1 to 349 amino acids) was cloned into pET28a vector (Novagen), 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 Asn hydroxylation of FITC-HIF-1α peptide (788 to 822 amino acids) (MW 4332.2) (fluorescein-5-isothiocyanate-*aca*-DESGLPQLTSYDCEVNAPIQGSRNLLQGEELLRAL) or FITC-HIF-1α peptide (786 to 826 amino acids) C800A (fluorescein-5-isothiocyanate-*aca*-SMDESGLPQLTSYD*A*EVNAPIQGSRNLLQGEELLRALDQVN) (MW 4975.3) (AnyGen, GwangJu, Korea) developed for another assay (Cho et al., 2007; Cho et al., 2005). The peptide was incubated at a final concentration of 4 μM with 0.7 μg of recombinant FIH-1 with 100 μM of α-ketoglutarate , 400 μM or 2 μM of 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).

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Mass Spectrometric Analysis — After hydroxylation for 2 h at room temperature, excess salts were removed from substrate peptides with $ZipTip_{C18}$ (Millipore). 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).

Western blot analysis and co-immunoprecipitation — 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 previously described (Choi et al., 2006b). For immunoprecipitation, 200 μg of whole cell lysate was incubated with 1 μg of anti-CBP antibody (Santa Cruz Biotechnology) at 4□ overnight. The resulting immunocomplexes were analyzed by Western blotting with anti-human HIF-1α antibody (BD Biosciences) (Choi et al., 2006b).

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

Northern analysis and reverse transcriptase PCR (RT-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

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VEGF as described previously (Yim et al., 2003). RT-PCR was performed with total RNA (1 μg), and 1 μM of each primer 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) were used. We used the following primers; human VEGF, F5'-AACCATGAACTTTCTGCTGTCTTG-3' and R5'-TTCACCACTTCGTGATGATTCTG-3' ; human CA9, F5'-CAGTTGCTGTCTCGCTTGGA-3' and R5'-TGAAGTCAGAGGGCAGGAGTG-3' ; 18S, F5'-ACCGCAGCTAGGAATAATGGAATA-3' and R5'-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.

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RESULTS

NOC18-, spermine NONOate-, and SNAP-induced stabilization of HIF-1α — In order 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, upper panel). Based on these results, the following experiments focused on spermine NONOate and SNAP. We found that 2 hour treatments with spermine NONOate or SNAP maximally increased the level of HIF-1α, and that 200 μM of spermine NONOate and 100 μM of SNAP maximally raised HIF-1α in normoxic HeLa cells to levels even higher than in hypoxic (1% O_2) cells. We then showed that the NO donors lead to the expression of target genes such as Vascular Endothelial Growth Factor (VEGF) and carbonic anhydrase-9 (CA9) after 2 to 3 hour 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 — In order 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. Previously we found that Zn 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 cotransfected HA-tagged Ubiquitin and Flag-tagged HIF-1α and then performed co-immunoprecipetation assay by using anti-HA antibody. We confirmed that SNAP reduced the ubiquitinated form of HIF-1α (Fig. 2B). O_2 dependent ubiquitination of

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HIF-1α is mediated by an HIF-1α-specific E3 ligase named VHL. Since VHL specifically recognizes and binds to the hydroxylated proline residues of the ODD domain (401-603 amino acids), 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 purified GST-HIF-1α (401-603 amino acids) fusion protein as substrate. Since we used the glutathione resin-bound form of GST-HIF-1α (401-603 amino acids), glutathione was not present in the subsequent reactions. GST-HIF-1α (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 μM 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 non-enzymatic 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 (Li et al., 2007; Sumbayev et al., 2003). Previously, we showed that 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. Interestingly, 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, while 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

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confirm these findings, we treated HeLa cells with vitamin C (100 μM) and SNAP (200 μM) for 4hr. The results in Fig. 3D show that both vitamin C and Fe(II) abolish the effect of SNAP on HIF-1α stability, while 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 — In order to see whether SNAP inhibits the hydroxylation reaction or VHL recruitment, we directly assessed its effect on hydroxylation of Pro-564 of HIF-1α by measuring changes in the molecular weight of HIF-1α peptide (556-575 amino acids) by mass spectroscopy. For the hydroxylation reaction we used purified his-tagged PHD2 (184-418 amino acids), 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 HIF-1α peptide (556-575 amino acids) in the hydroxylation-dependent VHL pull down assay instead of GST-HIF-1α (401-603 amino acids). The ODD domain of HIF-1α contains one cysteine residue (residue 520) but the HIF-1α peptide contains no cysteine. PHD2 increased 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 (556-575) with VHL in the presence of SNAP, confirming that the cysteine residue of HIF-1α-ODD but not of GST mediate the effect of SNAP (supplemental data).

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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 C520 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 C520 presumably prevents hydroxylated HIF-1α from recruiting VHL.

Effects of SNAP on Asn hydroxylation and CBP binding of HIF-1α — To induce transactivation capability, the stabilized HIF-1α needs to recruit a co-activator, 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. In order to test the effect of SNAP on FIH-1 activity we incubated 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-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. 6B). Thus the, Asn-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. In order 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

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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 Asn-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 also the transactivation of HIF-1α by inhibiting both VHL recruitment and Asn-hydroxylation, we tested by co-immunoprecipitation whether HIF-1α stabilized by SNAP was able 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.

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DISCUSSION

We have confirmed that SNAP stabilizes the *trans*-active 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 from 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 co-treated 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*. recently demonstrated, 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 (Li et al., 2007). They also demonstrated by a biotin switch assay, that Cys533 of mouse HIF-1α (equivalent to Cys-520 of human HIF-1α) is nitrosylated, and that this S-nitrosylation does not inhibit proline hydroxylation. By using hydroxylation dependent VHL pull 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 *trans*-active 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. Since 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 (786-826) peptide using reverse-phase HPLC instead of mass analysis, we confirmed that the purified HIF-1α (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). Since NO increases not only the stability but also the transactivation of HIF-1α, S-nitrosylation of Cys-800 is expected to increase its interaction with the coactivator CBP/p300 (Sumbayev and Yasinska, 2007; Yasinska and Sumbayev, 2003). Using a fluorescence polarization-based interaction assay (Cho et al., 2005), we found that S-nitrosylation of HIF-1α (786-826) peptide instead decreased the p300 interaction. In addition, SNAP inhibited the hydroxylation of Asn-803 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 Cys-800 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. Recently, the inhibitory effect of antioxidants including vitamin C (Gao et al., 2007; Lu et al., 2005) and Fe(II) (Gerald et al., 2004; Lu et al., 2005) on

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tumorigenesis has been reevaluated in the context of HIF-1α destabilization. Gao *et al*. reported that antioxidants such as N-acetylcysteine and vitamin C inhibit lymphoma xenografts by diminishing hypoxia-inducible factor (HIF)-1 levels in a prolyl hydroxylase 2- and von Hippel-Lindau protein-dependent manner (Gao et al., 2007). In addition, TCA 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 (Pan et al., 2007; Selak et al., 2005). Fumarate and malate are other TCA 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 reactive oxygen species (mtROS) are essential for proper O_2 sensing by PHDs. In *cyt* c null embryonic cells, which have defects in generating mtROS, 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, so stabilizing the trans-active form of HIF-1α in normoxic cells. NO is released in many patho-physiological conditions including inflammation and blood clotting (Li et al., 2007; Vadseth et al., 2004; 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 Asn-hydroxylation, leading to functional HIF-1α, and whether the NO effects can be modulated by other inflammatory mediators (Li et al., 2007).

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Footnotes

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Figure legends

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, 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 hours. (B) The mRNA level of VEGF (upper panel) was detected by Northern analysis and the mRNA level of carbonic anhydrase 9 (lower panel) by RT-PCR. By using quantitative real time PCR, measured were mRNAs of VEGF and CA9 of which HeLa cells were exposed to SNAP (200 μM), spermine NONOate (200 μM) or hypoxia for 6 hours. (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.

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, HIF-1α-ubiquitination blocker, TPEN (Choi et al., 2006a) for 4 hr under normoxic conditions. HIF-1α protein was detected by Western blot analysis. HIF-1α and ubiquitinated HIF-1α are indicated. *N* indicates normoxia, and *H* indicates hypoxia (1% oxygen). (B) 293 cells were transfected with Flag-HIF-1α, HA-ubquitin as indicated. The transfected cells were treated with SNAP, MG132 or hypoxic mimicker, $CoCl_2$ for 4 hr 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 protein was detected to confirm equal loading.

Fig. 3. Effects of SNAP on HIF-1α-proline hydroxylation-dependent VHL recruitment. Resin-bound GST-HIF-1α(401-603) was incubated with recombinant his-tagged PHD2 (amino acid 184-418) (1 μg) in the presence or absence SNAP (A) with 100 μM Fe(II), 2mM vitamin C, and 5mM α-ketoglutarate (α-KG); (B) with 2mM vitamin C, and 5mM α-KG; (C) with only 5mM α-KG. After the hydroxylation reaction, resin-bound GST-HIF-1α(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 (401-603) protein stained with Coomassie blue.

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The first lane represents ten percent 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 hr. HIF-1α was detected by Western blot analysis. HSP70 was detected by Western blot analysis for loading control.

Fig. 4. Effect of SNAP on proline-hydroxylation of HIF-1α (A) 2 μM biotin-HIF-1α-(556-575) peptide (MW 2637.0) and 100 μM of α-KG were incubated together with the following combinations of the three reagents 1 μg of recombinant his-PHD2 (184-418aa), 400 μM of vitamin C, SNAP as indicated., Fe(II) was not included. The reaction mixtures were analyzed by MALDI-TOF as described (Cho et al., 2007). The detected mass of the major peptide was shown above the peak. (B) 2 μg of biotin-HIF-1α(556-575) peptide (MW 2637.0) was hydroxylated in the absence or presence of SNAP, by adding recombinant his-PHD2 (184-418aa) (1 μg) and 400 μM α-KG. Biotin-HIF-1α(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(556-575) peptide was visualized by SDS-PAGE and autoradiography. The first lane represents ten percent of the [^{35}S]-VHL used.

Fig. 5. Effect of SNAP and cysteine 520 of HIF-1α. (A) Resin-bound GST-HIF-1α(401-603) was incubated with his-PHD2 (184- 418) (1 μg) and 5mM 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(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(401-603) protein stained with Coomassie blue. The first lane represents ten percent of the [^{35}S]-VHL used. (B) Resin-bound GST-HIF-1α(401-603) C520A mutant protein was used instead of GST-HIF-1α(401-603).

Fig. 6. Effects of NO donors on asparagine hydroxylation of HIF-1α (A) FITC-HIF-1α-(788-822) peptide (MW 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-(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 ºC for 2 hr, followed by MALDI-TOF analysis. Note that the indicated molecular weights correspond to the peptides with detached FITC (MW 389.0) in a sodium (MW 23.0)-added form (MW 3965.7) during the MALDI-TOF measurements. The detected mass of the major peptide was shown above

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the peak.

Fig. 7. Effect of SNAP and cysteine 800 of HIF-1α. (A) In the presence or absence of SNAP (500 μM), 3 μM of FITC-HIF-1α-(788-822) peptide (upper panel) or 3 μM of FITC-HIF-1α-(786-826) C800A peptide (MW 4975.3) (lower panel) was incubated along with his-FIH-1 (1 μg) with α-KG (100 μM) and vitamin C (2 μM) at 30 ºC for 2 hr, followed by MALDI-TOF analysis (Cho et al., 2007). Note that the indicated molecular weights correspond to the peptides with detached FITC (MW 389.0) in a sodium (MW 23.0)-added form (MW 4608.8) during the MALDI-TOF measurements. The detected mass of the major peptide was 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 hr. 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 protein was detected with anti-HSP70 antibody.

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

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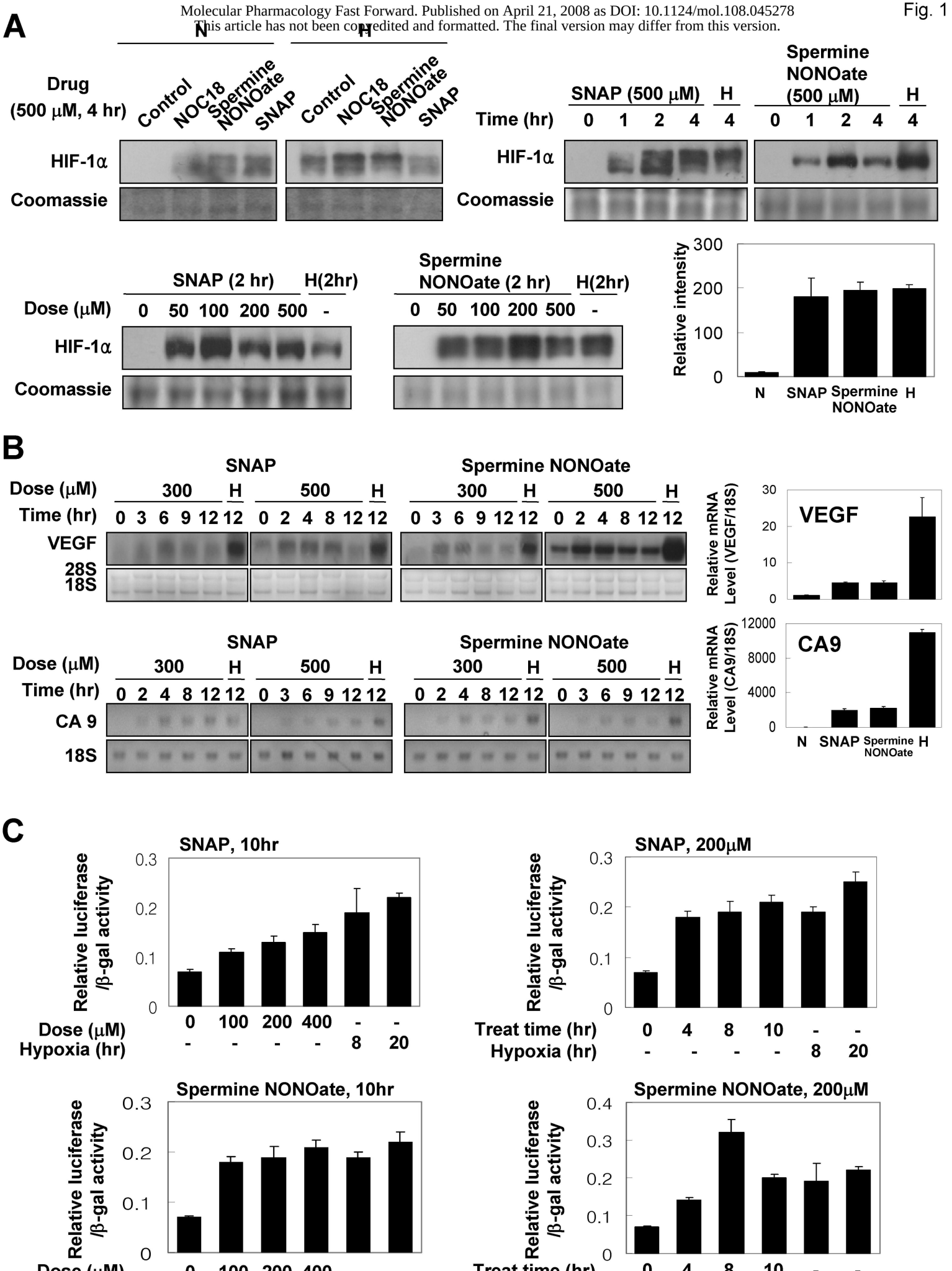
Fig. 1



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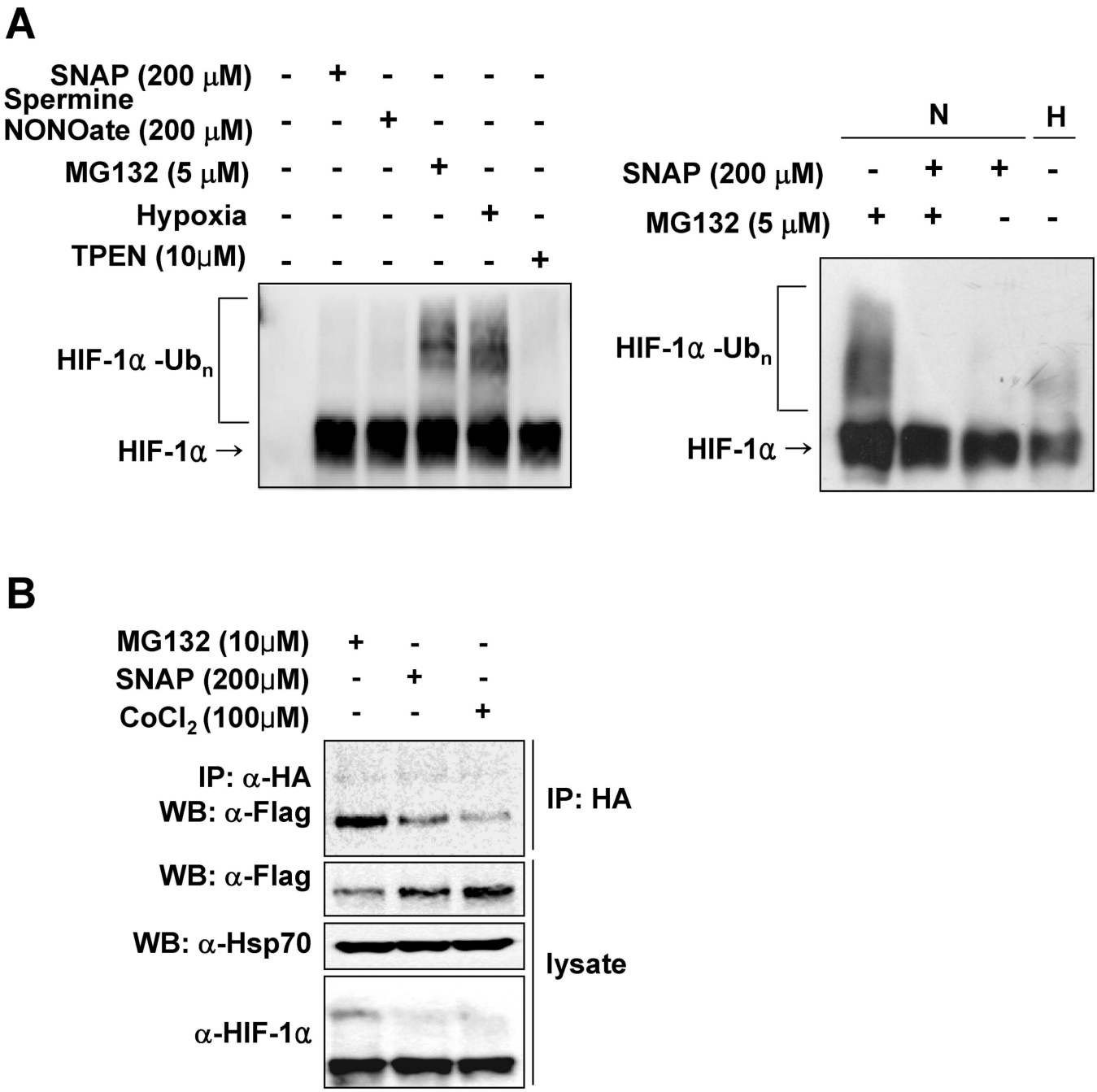

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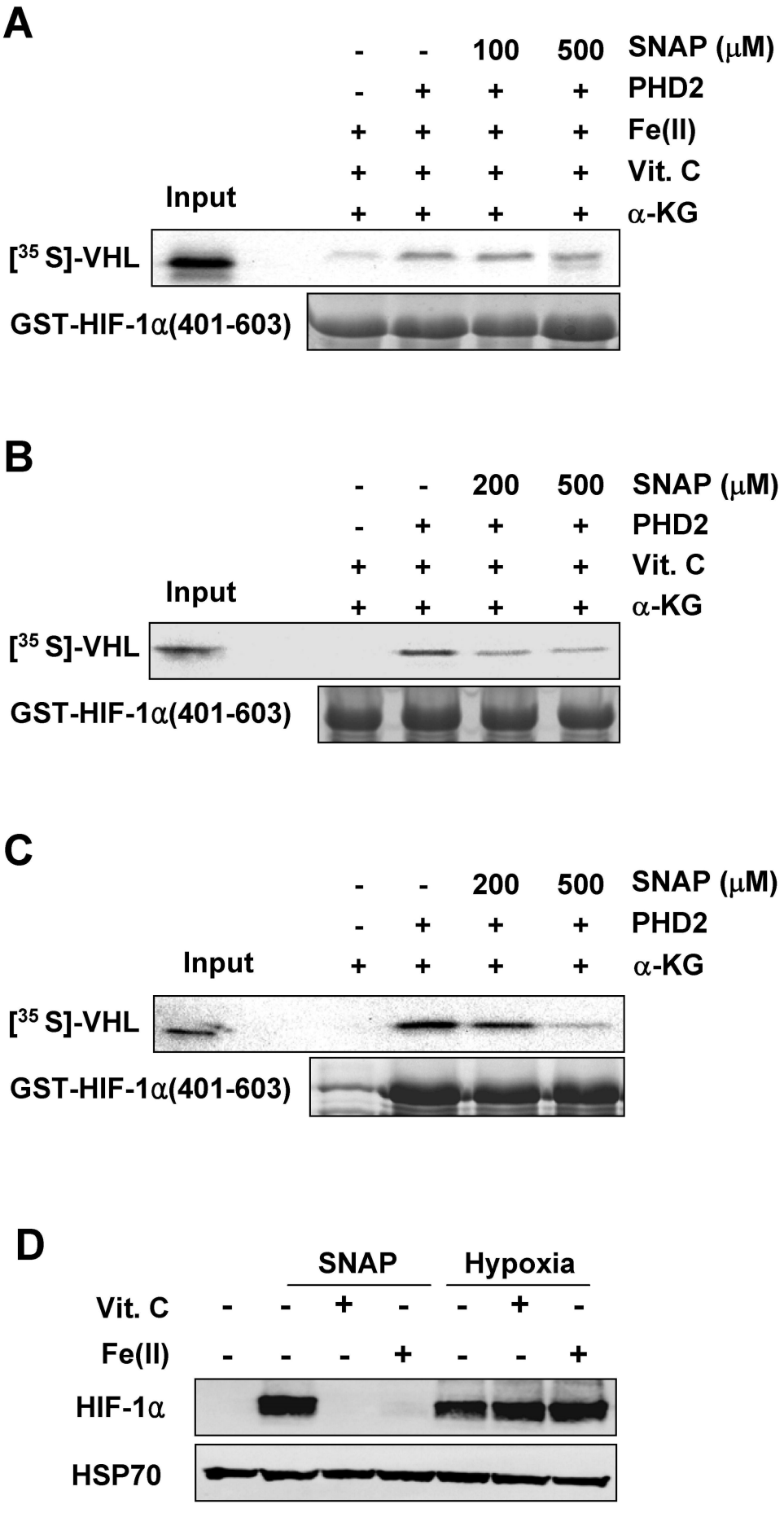

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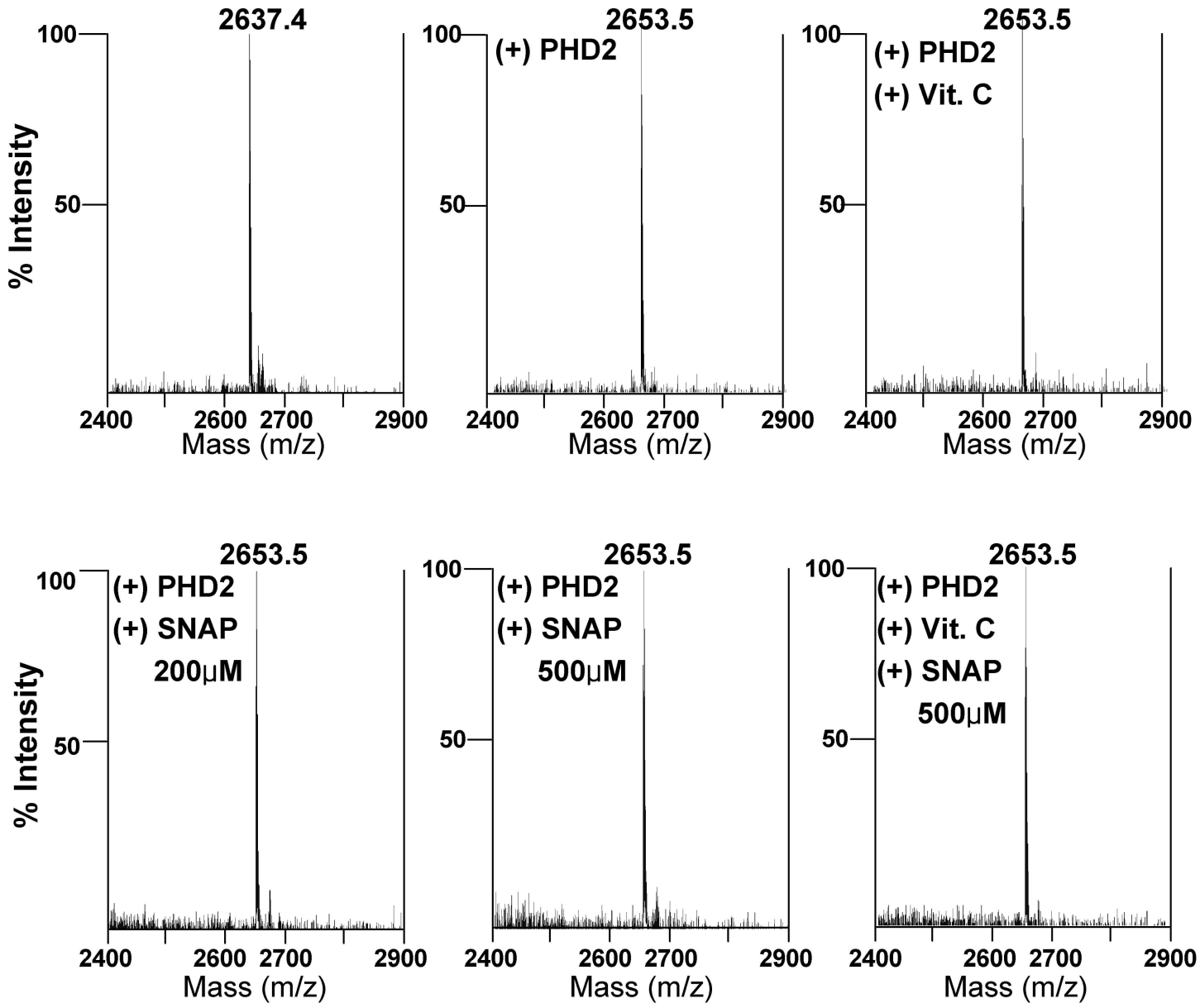

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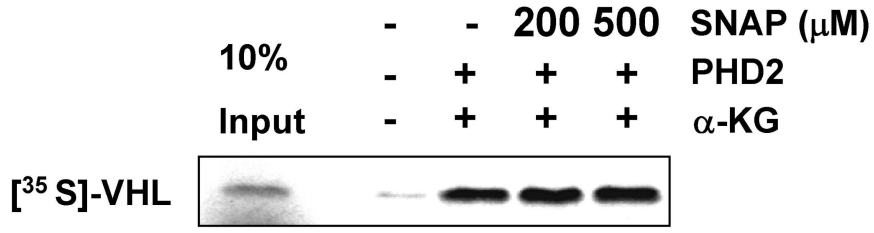

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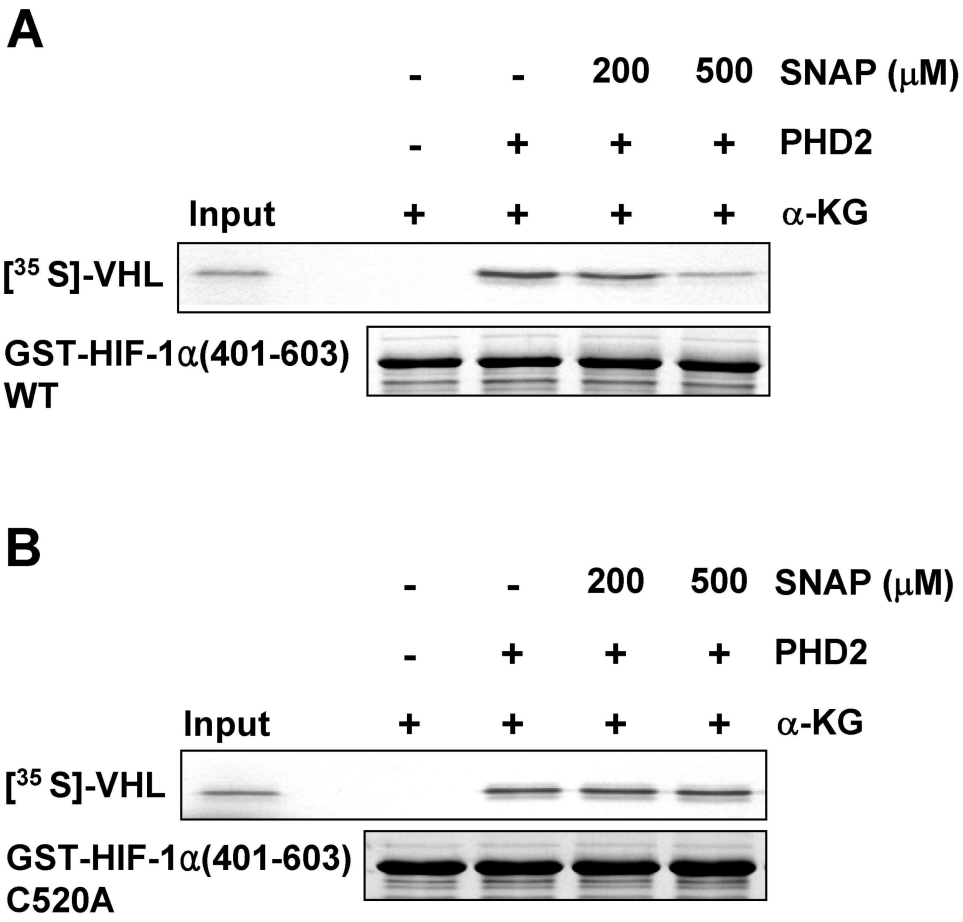

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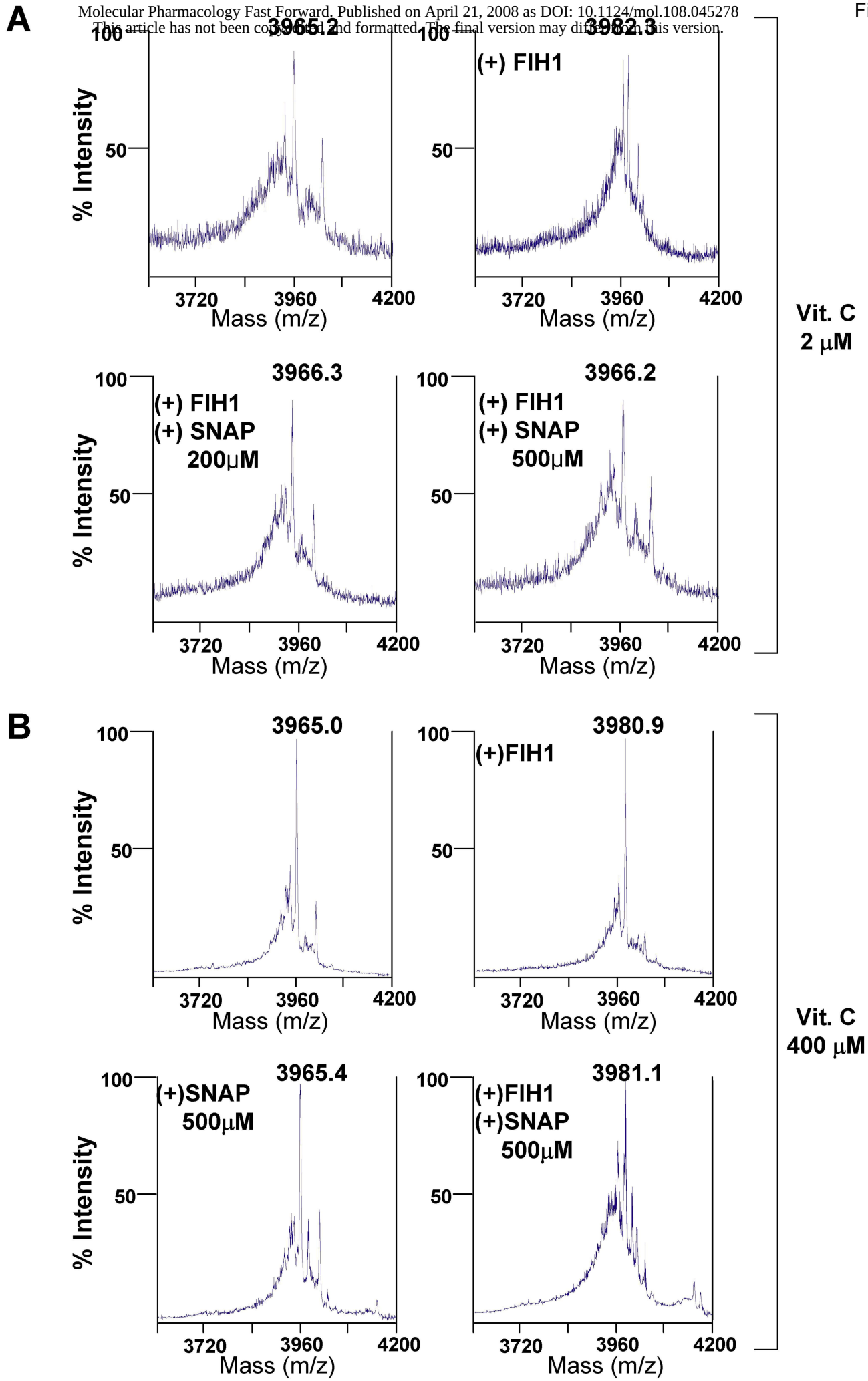
Fig. 6



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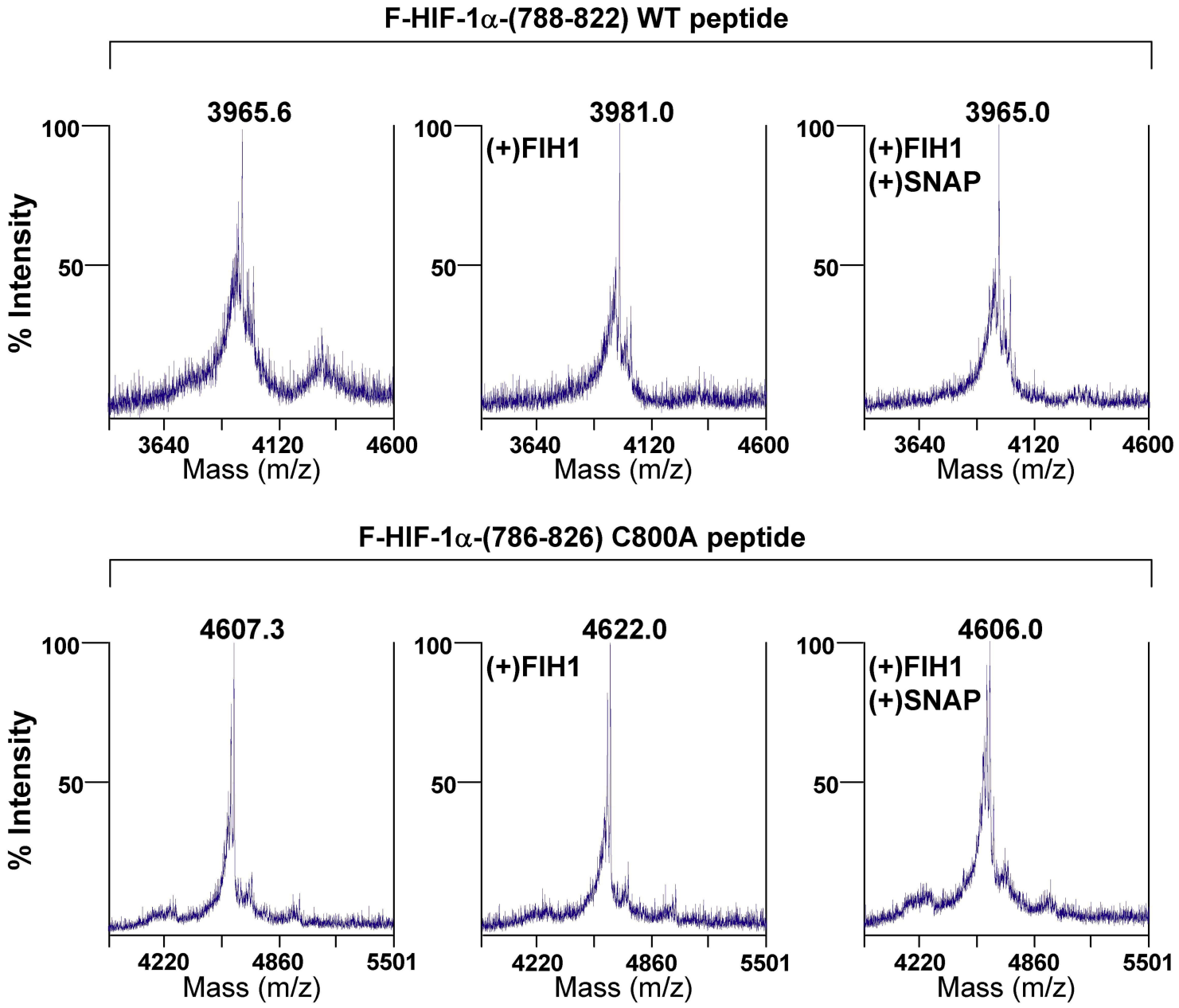

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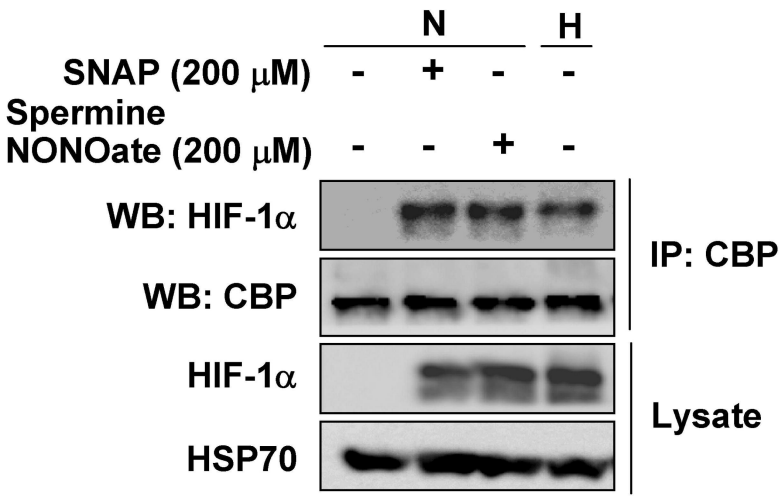

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