Cobalt Inhibits the Interaction between Hypoxia Inducible Factor-α and von Hippel-Lindau

Protein by Direct Binding to Hypoxia Inducible Factor-α

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Abstract

The hypoxia-inducible factor (HIF) activates the expression of genes that contain a hypoxia response element (HRE). The alpha subunits of the HIF transcription factors are degraded by proteasomal pathways during normoxia, but are stabilized under hypoxic conditions. The von Hippel-Lindau protein (pVHL) mediates the ubiquitination and rapid degradation of HIF-α (including HIF-1α and HIF-2α). Post-translational hydroxylation of a proline residue in the oxygen dependent degradation (ODD) domain of HIF-α is required for the interaction between HIF and VHL. It has previously been established that cobalt mimics hypoxia and causes accumulation of HIF-1α and HIF-2α. However, little is known about the mechanism by which this occurs. In an earlier study, we demonstrated that cobalt binds directly to the ODD domain of HIF-2α. Here we provide the first evidence that cobalt inhibits pVHL binding to HIF-α even when HIF-α is hydroxylated. Deletion of 17 amino acids within the ODD domain of HIF-2α that are required for pVHL binding prevented the binding of cobalt and stabilized HIF-2α during normoxia. These findings show that cobalt mimics hypoxia, at least in part, by occupying the VHL-binding domain of HIF-α and thereby preventing the degradation of HIF-α.

Introduction

Hypoxia is a critical stimulus in many physiological and disease states (1). Cells respond to hypoxia by regulating the expression of a number of genes, including erythropoietin, vascular endothelial growth factor and various glycolytic enzymes (2, 3, 4 and 5). This regulation is mediated in part by transcription factors of the hypoxia-inducible factor (HIF) family (6). HIF-1α and HIF-2α are basic helix-loop-helix PAS domain (bHLH-PAS) proteins (7) that form a heterodimer with the aryl hydrocarbon nuclear receptor translocator (ARNT) protein. Previous studies have shown that HIF-1α protein accumulates rapidly during hypoxia without a significant increase in HIF-1α mRNA levels (8). HIF-2α, which is also known as endothelial PAS domain protein-1 (EPAS1), shares close sequence and structural homology with HIF-1α (9). Like HIF-1α, the levels of HIF-2α protein are low during normoxia and accumulate when cells are exposed to hypoxia, proteasomal inhibitors, transition metals (e.g. cobalt), iron chelators, or reducing agents (10). During normoxia, the HIF-α (HIF-1α and HIF-2α are referred to here simply as HIF-α, except where noted otherwise) proteins are continuously degraded by a ubiquitin- and proteasome-dependent pathway. Detailed studies of HIF-α proteins revealed a 200 amino acid sequence, called the oxygen-dependent degradation domain (ODD), which is responsible for its degradation in the presence of oxygen (11, 12). The von Hippel-Lindau (pVHL) protein, a tumor suppressor protein, mediates the ubiquitination and degradation of HIF-α by binding to the ODD domain under conditions of normoxia (13, 14). Recent findings revealed that pVHL-mediated degradation requires hydroxylation of specific proline residues within the ODD (15, 16, 17, and 18). The hydroxylation of these proline residues may be critical

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for regulating the HIF levels and therefore transcription of downstream hypoxia-responsive genes.

It has been well documented that cobalt, a transition metal, mimics hypoxia by causing the stabilization of HIF-α. However, the biochemical mechanism by which cobalt stabilizes HIF-α remains unknown. A recent model suggested that the hydroxylation of HIF-α is mediated by a group of HIF-specific hydroxylases and that cobalt can inactivate the enzymes by occupying iron-bind site on the proline hydroxylases (18). We previously reported that HIF-2α binds cobalt *in vitro* and that the cobalt-binding site overlaps with the pVHL-binding site on HIF-2α (19). Here we show that cobalt inhibits the interaction between pVHL and hydroxylated HIF-α and that cobalt inhibits the hydroxylation of a key proline residue within the ODD domain of HIF-2α. This is the first report that cobalt stabilizes cellular HIF-2α by occupying the VHL-binding domain.

Materials and methods

Cell culture and reagents. Rat pheochromocytoma (PC12) cells and CHO cells were obtained from the American Type Culture Collection (Manassas, VA) and Renal Clear Carcinoma (RCC)VHL^{+} cells were provided by Dr. Czyzyk-Krzeska (University of Cincinnati). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 20 mM HEPES, pH 7.4, 10% fetal bovine serum (Life Technologies, Inc.), penicillin (100 unit/ml), and streptomycin (100 µg/ml). The medium for RCC/VHL^{+} cells was supplemented with 200 µg/ml G418. Prior to experimentation, cells were grown to approximately 85% confluence in 100-mm tissue culture dishes (Corning Incorporated, Corning, NY). CoCl$_2$ was from Sigma. Reagents used for

hemagglutinin (HA) immunoprecipitation (anti-HA antibody and protein G/A plus-coupled agarose) were from Santa Cruz Biotechnology (Santa Cruz, CA). The HIF-2α antibody was from Novus Biologicals (Littleton, CO).

Plasmids. The HIF-1α expression vector (pcDNA3-HIF-1α) was a gift from Dr. Steven L. McKnight (University of Texas Southwestern, Dallas, TX). The HA-tagged pVHL plasmid pRC/CMV-HA-VHL was originally from Dr. William G. Kaelin Jr. (Harvard Medical School, Boston, MA). HIF-2α expression plasmids were constructed as described previously (19). pTriEx-HIF-2α (48-688), expresses amino acids 48 to 688 of HIF-2α, which includes the wild type ODD domain. pTriEx-HIF-2α (48-523,539-688) expresses HIF-2α with a deletion from amino acid 523 to 539, which forms the core of ODD domain and the pVHL-binding site.

Western Blots. HIF-2α western blots were performed as describe previously by Conrad et al. (20).

Binding Assays. HIF-VHL binding assays were performed as described by Cockman et al (21). Briefly, ^{35}S-labeled HIF-α and pVHL were generated in TNT reticulocyte lysates (Promega, Madison, WI), as described. Proteins were then mixed in binding buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.5% Igepal) in the presence or absence of transition metals at 4°C for 1 hr. Samples were then subjected to immunoprecipitation with an anti-HA antibody and analyzed by SDS-PAGE followed by autoradiography.

In vitro **degradation assay**. ^{35}S-labeled proteins (1 μl) were added to the following reaction: 30 μl RCC/VHL[+]cell cytoplasmic extract, 6 μl untreated rabbit reticulocyte lysate (Promega), 25 μg

of ubiquitin (Sigma), 5 µl of 10×ATP-regenerating system (20 mM Tris, pH 7.5,10 mM ATP, 10 mM magnesium acetate, 300 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase), and water for a final volume of 50 µl. Each reaction was incubated at 30°C, then stopped by the addition of SDS-PAGE loading buffer at the indicated times. The labeled proteins were analyzed by SDS-PAGE followed by autoradiography.

HIF-2α *In vitro* cobalt binding experiment

Fifty µl of HisBind Resin (Novagen) was charged with 1 ml of 50 mM $CoCl_2$. The charged resin was then washed and suspended in binding buffer (20mM Tris HCl, pH 7.4, and 500 mM NaCl). Cell extracts were prepared by snap-freezing cells in Cell Lysis Buffer (20mM Tris HCl pH 7.4, 150 mM NaCl, 0.5% TritonX-100). Cell extracts containing 500 µg of protein were mixed with resin and incubated at 4°C for 15 min, then washed with 1 ml of binding buffer. The proteins were washed and eluted with binding buffer containing 60 mM imidazole, followed by elution with 1 M imidazole. The remaining uneluted proteins were solubilized with SDS-PAGE sample buffer and the resulting samples were subjected to western blotting for HIF-2α.

Mass Spectrometry: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) analysis was performed with a Bruker Biflex III equipped with reflector and delayed extraction. Sample preparation and MALDI analysis were modified according to Lehmann et al (22). Briefly, equal volumes of peptides (0.1 mM), matrix (35 mM ATT in 10 mM Tris, pH 9.0) and metal chloride solution (1.0 mM) were mixed and spotted onto the MALDI target. The extraction and reflector settings were 19 kV and 20 kV respectively, and laser attenuation was set to 54%. The resulting spectra were summed over 300 shots. The HIF peptide was synthesized by Bio-Synthesis, Inc. (Lewisville, TX). The sequence of the HIF

peptide is shown in Fig. 3A. The control peptide (adrenocorticotropic hormone fragment 18-39, ACTH), and the matrix 6-aza-2 thiothymine (ATT) were from Sigma-Aldrich.

Results

The oxygen-dependent proteolytic destruction of HIF-α is mediated by the von Hippel-Lindau protein (pVHL), which is an E3 ubiquitin ligase (13). The binding of pVHL to HIF-α is regulated through the hydroxylation of a proline residue (HIF-1α P564 and HIF-2α P531) in the ODD domain of HIF-α (14, 15). The hydroxylation of this proline requires active cell extracts, iron and oxygen (18). Figures 1A and 1B show that HIF-1α and HIF-2α translated *in vitro* using reticulocyte lysates in the presence of excess iron (100 μM $FeCl_2$) enhanced pVHL-binding activity. Iron chelators, such as desferrioxamine (DFO) or dipyridyl (DIP), and the transition metal cobalt chloride stabilize HIF-α during normoxia. Experiments were designed to determine if these hypoxia-mimicking reagents stabilize HIF-α by inhibiting iron-mediated proline hydroxylation of HIF-α. Unhydroxylated HIF-2α was translated *in vitro* in the absence of excess iron. The hydroxylation reaction was performed in cell extracts from RCC-VHL$^+$ cells. This cell line was not selected for any special reason; in fact, extracts from most mammalian cell lines can provide the activity required for this hydroxylation reaction. Supplementary iron (10 μM $FeCl_2$) substantially increased pVHL binding to HIF-2α, as detected by the pVHL-binding assay, which indicates enhanced hydroxylation of HIF-2α (Figure 1C). When cobalt (100 μM $CoCl_2$) or iron chelators (100 μM DFO or DIP) were present, the inhibition of iron-mediated hydroxylation of HIF-2α resulted in decreased binding of HIF-2α to HA-VHL (Figure 1C). Thus, both cobalt and iron chelators inhibit the iron-mediated hydroxylation of HIF-2α.

We next examined the effects of iron chelators and cobalt on the HIF-VHL interaction. HIF-1α and HIF-2α were translated in reticulocyte lysate in the presence of iron (100 μM $FeCl_2$), which leads to proline hydroxylation of HIF-α (15). The translation products and HA-VHL were added to the binding buffer. In some cases, cobalt (100 μM $CoCl_2$) or the iron chelator (DFO 100 μM) was also added to the binding buffer. Results from these experiments showed that the addition of cobalt (100 μM) greatly reduced the interaction between hydroxylated HIF-α and pVHL, whereas DFO had little effect (Fig. 2A and 2B). We next performed a series of experiments to determine the optimal concentration of cobalt required to inhibit the HIF-VHL interaction. To do this, we used *in vitro* translation to generate ^{35}S-methionine-labeled and hydroxylated HIF-1α and HIF-2α in the presence of iron (100 μM $FeCl_2$). We then added various concentrations of $CoCl_2$ to the binding reactions. We found that cobalt inhibited the interaction between HIF-α and pVHL in a concentration-dependent manner and that the maximal inhibition occurred at 100 μM $CoCl_2$ (Figures 2C and 2D). Western blot analysis revealed that the same concentration of cobalt was required to stabilize HIF-2α in PC12 cells (Figure 2D; lower panel). These findings show that the level of cobalt required to inhibit the HIF-VHL interaction *in vitro* are similar to the concentration needed to stabilize HIF-2α within PC12 cells. These results suggest that iron chelators stabilize HIF by inhibiting iron-mediated hydroxylation of the proline residue of HIF. In contrast, cobalt not only inhibits hydroxylation, but also directly inhibits the HIF-VHL interaction.

Experiments were next performed to determine the mechanism by which cobalt inhibits the HIF-VHL interaction. We previously reported that HIF-2α binds to cobalt *in vitro* and that cobalt and VHL bind to the same site within the ODD (18). The 17 amino acid cobalt-binding region

contains three glutamic acid residues (E) and three aspartic acid residues (D) (Figure 3A). The carboxyl side groups of these acidic amino acids provide possible sites for cobalt binding. When HIF-2α was mutated by removing this 17 amino acid fragment (Figure 3A), the resulting ODD⁻ mutant had a prolonged half-life, as determined by *in vitro* degradation experiments (Figure 3B). Moreover, the pVHL binding activity was abolished in the ODD⁻ HIF-2α protein (Figure 3C). The ODD⁻ mutant HIF-2α also lacked cobalt-binding activity (Figure 3D). These data strongly suggest that cobalt competes with pVHL for binding to HIF-α. To obtain direct evidence that cobalt binds to HIF-α, we next used matrix-assisted laser desorption time-of-flight (MALDI-TOF) to analyze the interaction of cobalt and a synthetic peptide that consisted of the deleted amino acid sequence shown in Figure 3A (amino acids 523-539 of HIF-2α). We found that this peptide binds directly to cobalt, whereas a control peptide (ACTH) that also contained five acidic amino acids did not bind to cobalt (Figure 4). This confirms that cobalt binds directly to a specific amino acid sequence within the ODD of HIF-α.

Discussion

The most recent model for oxygen sensing suggests that iron-mediated hydroxylation occurs via a group of HIF-specific proline hydroxylases (Figure 5A) (18). It was suggested that these hydroxylases have an iron binding center and that iron is critical for its enzymatic activity. Epstein et al. (18) further proposed that iron chelators can remove iron from the iron-binding center of the enzyme and that the iron can be replaced by cobalt at this site, which inactivates the hydroxylase activity. This model is consistent with the observations that cobalt and iron chelators inhibit hydroxylation of HIF. The present study demonstrates, for the first time, that cobalt also stabilizes HIF-α proteins by binding directly to the ODD and that cobalt inhibits both hydroxylation and the interaction between hydroxylated HIF-α and pVHL (Fig. 5B). This conclusion is supported by our MALDI data, which revealed that cobalt binds directly to a synthetic HIF peptide with a non-hyroxylated proline residue. In addition, our binding results show that cobalt inhibits the HIF-VHL interaction even after the proline residue becomes hydroxylated. Thus, cobalt can bind to HIF regardless of the hydroxylation state of the proline residue within the ODD.

DFO and cobalt appear to stabilize HIF-α via different mechanisms. Iron chelation effectively stabilizes HIF-α only when DFO is added to the translation reaction. The addition of DFO to modified HIF-α in the binding reaction did not prevent the association of HIF-α with VHL. Therefore, DFO most likely inhibits hydroxylation of HIF-α by chelating the iron required for activity of the HIF-specific proline hydroxylases. In contrast, cobalt prevents VHL binding when added at either the translation or the binding step. When cobalt was added during *in vitro* translation of HIF-α, the final concentration of cobalt in the HIF-VHL binding reaction was 0.5

μM. This is far below the concentrations of cobalt required to inhibit binding of hydroxylated HIF-α to VHL (50 to 100 μM). Thus, it is likely that cobalt interferes with multiple steps of the HIF-α degradation process (see Figure 5B). When added to the *in vitro* translation reaction, it is likely that cobalt prevents hydroxylation of HIF-α by binding to either HIF-specific proline hydroxylases or HIF-α itself. When cobalt was added to the binding reaction, it could still bind to hydroxylated HIF-α to prevent the interaction between HIF and VHL.

The present studies were carried out *in vitro*, and do not necessarily reflect how cobalt functions within cells. One possible model to explain how cobalt could stabilize HIF-α in cells is illustrated in Figure 5B. Cobalt may occupy the iron center of a HIF-specific proline hydroxylase, thereby inactivating the enzyme. Even if a subset of HIF-α did undergo hydroxylation, hydroxylated HIF-α could still bind to cobalt. Cobalt could thereby prevent the interaction between pVHL and hydroxylated HIF-α, which would prevent subsequent ubiquitination and degradation of HIF-α. Clearly, further studies will be needed to test this and other hypotheses, and to characterize the effects of cobalt *in vivo*.

Because the HIF-α transcription factor plays a critical role in the cellular response to changes in oxygen levels, it is not surprising that cells have evolved multiple mechanisms to regulate its activity. Three residues have been reported to be hydroxylated under normoxic conditions, including P402, P564 and N803 (15, 16, 17, 23). HIF-α may become oxidized (hydroxylated) at multiple sites under normoxic conditions to regulate its stability and activity. We have demonstrated that cobalt inhibits hydroxylation and pVHL binding of HIF-α at the P564 site. In

future studies, it will be important to determine whether cobalt similarly regulates HIF-α at the other two hydroxylation sites.

Acknowledgments

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Figure Legends

Fig.1. Cobalt inhibits iron-mediated hydroxylation of HIF-2α

Iron supplementation facilitates the interaction between pVHL and HIF-α. (A) ^{35}S-labeled HIF-1α and (B) HIF-2α were generated in reticulocyte lysates, in the presence or absence of Fe^{2+}, and then mixed with HA-VHL in a binding reaction, as described in Materials and Methods. The interactions were detected by anti-HA immunoprecipitation and analyzed by SDS-PAGE followed by autoradiography. (C) Cobalt and iron chelators inhibit iron-mediated hydroxylation of HIF-2α as assayed by the interaction between HIF-α and VHL. HIF-2α was produced in rabbit reticulocyte lysate, as described. Hydroxylation of HIF-2α was performed in extracts from RCC/VHL$^+$ cells in the presence or absence of iron (10 μM) alone, or with either cobalt (100 μM), or the iron chelators desferrioxamine (DFO, 100 μM) or dipyridyl (DIP, 100 μM). Reactions were incubated at 30°C for 60 min. HIF-VHL binding reactions were performed in binding buffer by mixing modified HIF-2α and HA-VHL, as described in Materials and Methods. The interactions were detected by anti-HA immunoprecipitation and analyzed by SDS-PAGE followed by autoradiography.

Fig. 2. Cobalt directly inhibits pVHL and HIF-α interaction

^{35}S-labeled and hydroxylated (A) HIF-1α and (B) HIF-2α were generated in reticulocyte lysates in the presence of Fe^{2+} (100 μM), then mixed with pVHL-HA in a binding reaction in the presence of either cobalt (100 μM) or DFO (100 μM), as indicated. The interactions were detected by anti-HA immunoprecipitation and analyzed by SDS-PAGE followed by

autoradiography. Labeled and hydroxylated HIF-1α (C) and HIF-2α (D) were generated in reticulocyte lysates, in the presence Fe^{2+} (100 μM). HIF-α proteins were then mixed with HA-VHL in binding buffer. The binding buffer included different concentrations of CoCl$_2$, as indicated. The interactions were detected by anti-HA immunoprecipitation and analyzed by SDS-PAGE followed by autoradiography. Lower blot in panel (D): PC12 cells were treated with increasing concentrations of cobalt chloride, as indicated, for 4 hr; HIF-2α protein levels were detected by western blotting.

Fig. 3. Cobalt inhibits the interaction between HIF-α and VHL interaction by occupying a conserved region of the ODD domain. (A) Schematic illustration of the HIF-2α constructs used in the experiment. ODD^{+} HIF-2α (upper construct) has an intact ODD domain, whereas ODD^{-} HIF-2α (lower construct) has a 17 amino acid deletion (523-539), which corresponds to the pVHL binding region of HIF-2α. (B). *In vitro* degradation of the ODD^{+} and ODD^{-} HIF-2α proteins. Labeled proteins were produced in reticulocyte lysates. Degradation reactions were performed as described in Materials and Methods, and reactions were stopped at the indicated times. C) ODD^{-} HIF-2α lacks pVHL binding activity. HIF-2α proteins were produced by reticulocyte lysates in the presence of iron (100 μM FeCl$_2$), then mixed with HA-VHL in the binding reaction. Interactions were detected by anti-HA immunoprecipitation and analyzed by SDS-PAGE followed by autoradiography. (D) ODD^{-}HIF-2α lacked the ability to bind to cobalt *in vitro*. Plasmids pTriEx-HIF-2α(48-688) and pTriEx-HIF-2α(48-523,539-688) were transfected into CHO cells and cells were then treated with 10 μM CbzLLL, a proteasomal inhibitor. The resulting cell extracts were then used in cobalt-binding assays. Cobalt-binding reactions were performed as described in Materials and Methods. In each experiment, lane 1 is

loading, lane 2 is run through, lane 3 is 60 mM imidazole elution, lane 4 is 1 M imidazole elution and lane 5 is SDS-PAGE sample buffer elution. The arrow indicates the amount of HIF-2α that remained bound to the resin after 1 M imidazole washing.
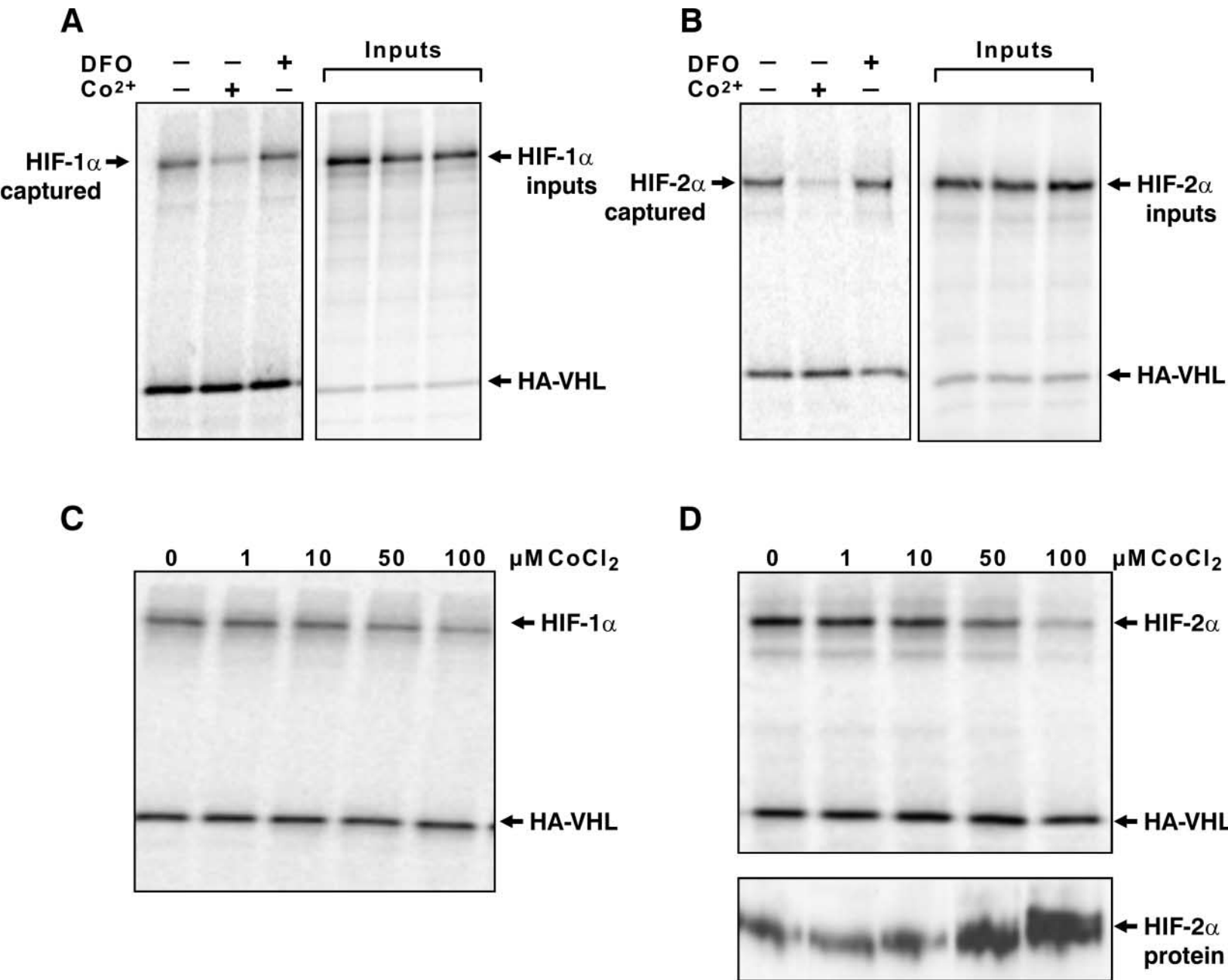
Fig.4. Matrix-assisted laser desorption time-of-flight (MALDI-TOF) analysis of HIF and ACTH peptides and their interactions with cobalt. A) The calculated monoisotopic MW of HIF peptide alone is 2349.1086 Dalton. The monoisotope peaks for HIF $[M+H]^+$ of 2350.702 m/z and contaminating sodium $[M+Na]^+$ and potassium $[M+K]^+$ adduct peaks of 2372.762 and 2388.662 m/z, respectively, are labeled. B) The calculated monoisotopic MW of ACTH peptide alone is 2464.191 Dalton. ACTH was used as a calibration and control peptide and its $[M+H]^+$ is shown. C) HIF peptide plus cobalt at a molar ratio of 1:10. There are four labeled monoisotopic peaks: $[M+H]^+$ at 2350.392 m/z, sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts at 2372.305 and 2388.369 m/z, respectively, and HIF peptide binding cobalt $[M-H+Co^{2+}]^+$ at 2407.388 m/z. D) ACTH peptide plus cobalt at a molar ratio of 1:10. The ACTH $[M+H]^+$ peak remained unchanged in the presence of cobalt. The mass spectra were externally calibrated. (A.I. is absolute intensity).

Fig. 5. Proposed model for oxygen sensing. (A) The current model for oxygen-sensing. The HIF-specific hydroxylase is thought to be an iron-binding protein. In the presence of oxygen, it catalyzes the hydroxylation of proline 564 within HIF. Hydroxylated HIF is then ubiquitinated by pVHL. Ubiquitinated HIF is degraded by proteasomal mechanism. Cobalt inhibits the hydroxylation of HIF by binding to the iron-binding domain of HIF hydroxylase. (B) Revised model based on our new *in vitro* data. Hydroxylation of HIF-α is mediated by a HIF-specific hydroxylase. Cobalt binds to the iron center of this enzyme to inactive the hydroxylase activity.
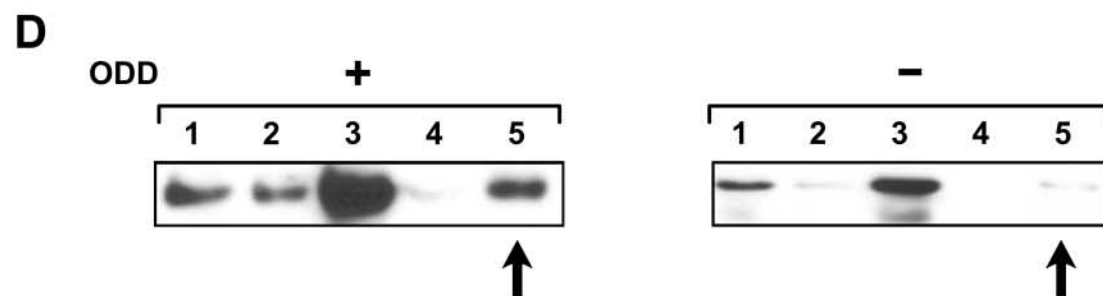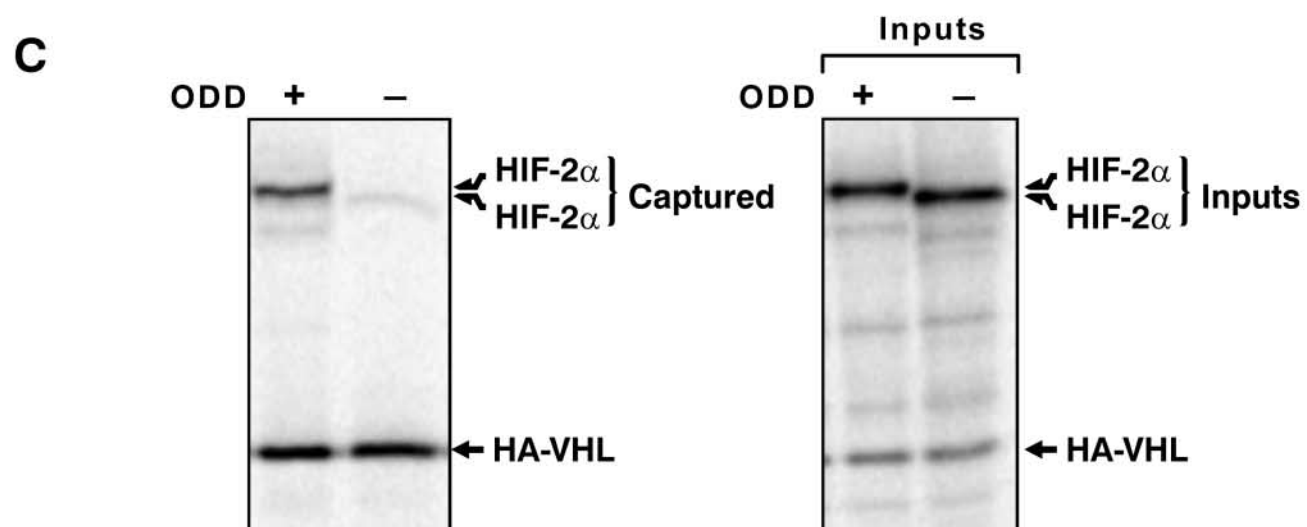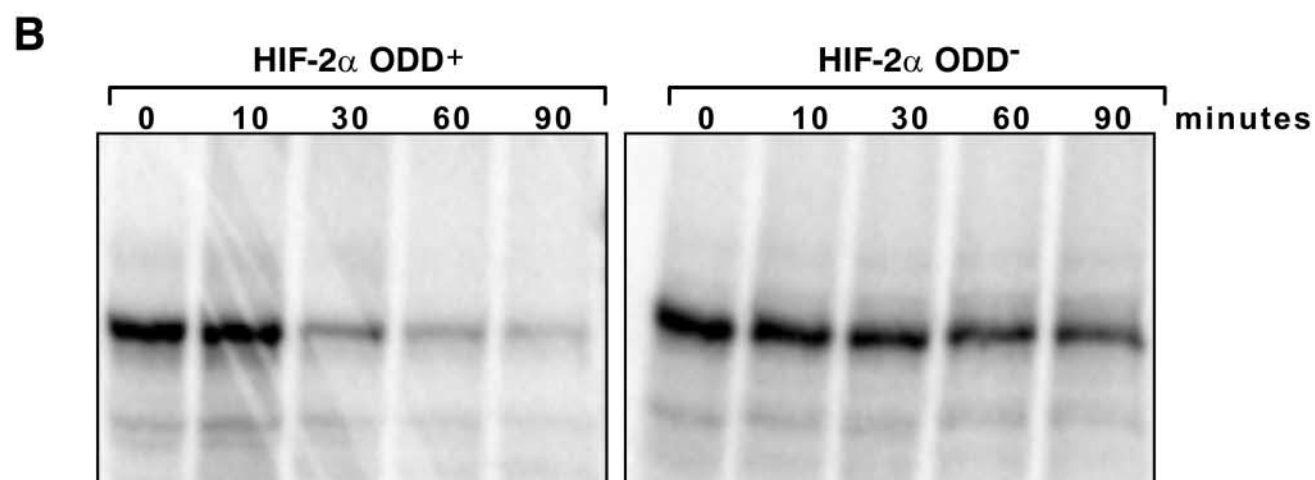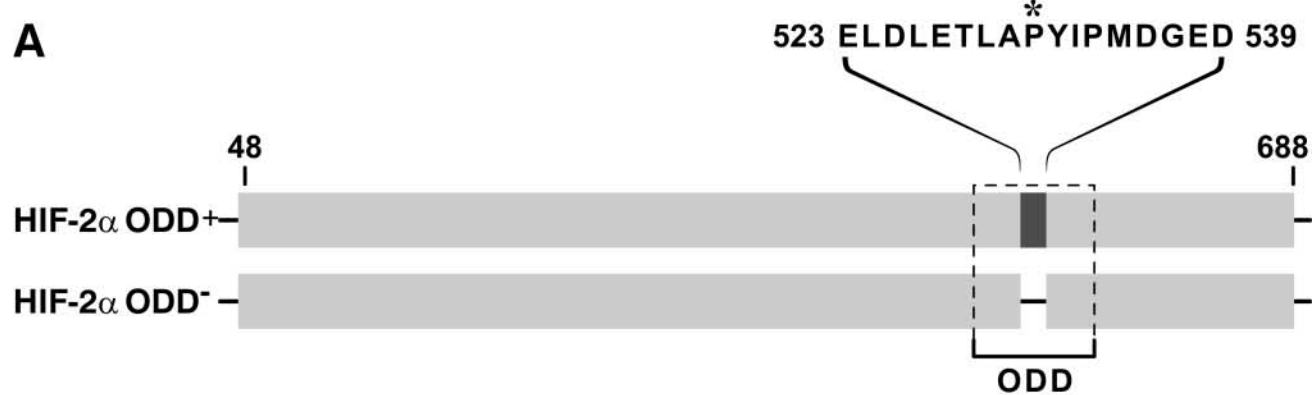
Even if a portion of HIF-α becomes hydroxylated, cobalt can also bind directly to the hydroxylated proteins to prevent the interaction between HIF-α and pVHL, thereby preventing the degradation of HIF-α.
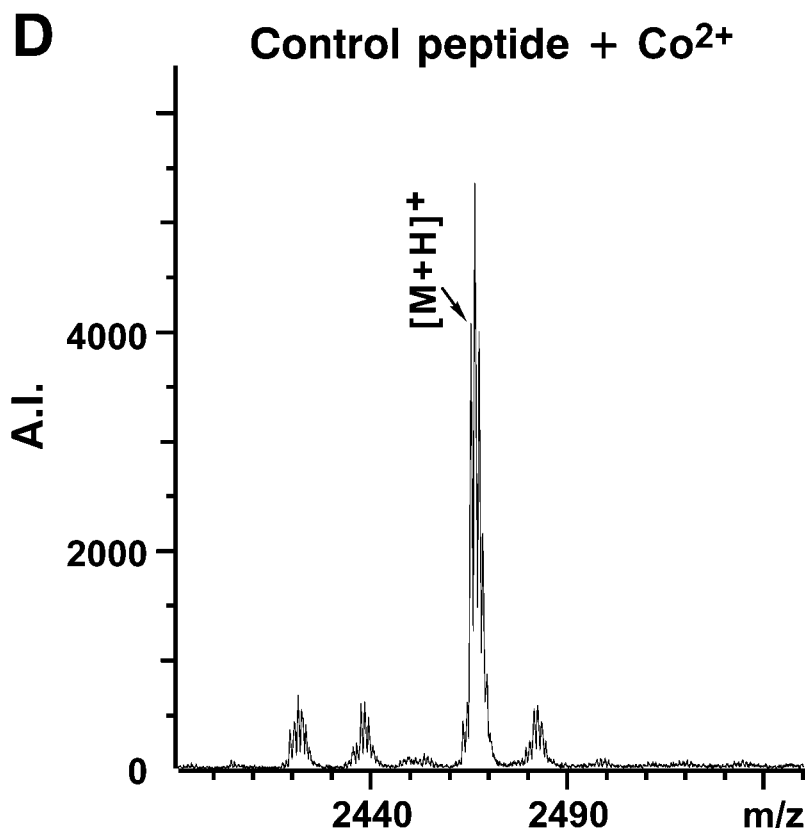
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Figure 1

A

DFO   −   −   +   Inputs

Co^{2+} −   +   −

HIF-1α →   ← HIF-1α
captured   inputs

← HA-VHL

B

DFO   −   −   +   Inputs

Co^{2+} −   +   −

HIF-2α →   ← HIF-2α
captured   inputs

← HA-VHL

C

0   1   10   50   100   µM CoCl$_2$

← HIF-1α

← HA-VHL

D

0   1   10   50   100   µM CoCl$_2$

← HIF-2α

← HA-VHL

← HIF-2α
protein

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Figure 2

A

523 ELDLETLAPYIPMDGED 539

48

HIF-2α ODD⁺

HIF-2α ODD⁻

688

ODD

B

HIF-2α ODD⁺   HIF-2α ODD⁻

0   10   30   60   90   0   10   30   60   90   minutes

C

ODD   +   −   ODD   +   −   Inputs

HIF-2α ⎤ Captured   HIF-2α ⎤ Inputs
HIF-2α ⎦   HIF-2α ⎦

HA-VHL   HA-VHL

D

ODD   +   −

1   2   3   4   5   1   2   3   4   5

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Figure 3

A HIF peptide
ELDLETLAPYIPMDGED

[M+H]⁺

[M+Na]⁺

[M+K]⁺

B Control peptide
RPVKVYPNGAEDESAEAFPLEP

[M+H]⁺

C HIF peptide + Co²⁺

[M+H]⁺

[M+Na]⁺

[M+K]⁺

[M-H+Co²⁺]⁺

D Control peptide + Co²⁺

[M+H]⁺

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Figure 5