Molecular Pharmacology Fast Forward. Published on April 18, 2008 as doi:10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #45690

BiPS, a matrix metalloprotease inhibitor, is a novel and potent activator of hypoxia-inducible factors

Marie-Claude Lauzier, Geneviève A. Robitaille, Denise A. Chan, Amato J. Giaccia and Darren E. Richard

Centre de recherche de L'Hôtel-Dieu de Québec and the Department of Medicine, Université Laval, Québec, QC, Canada (M.C.L., G.A.R., D.E.R.) and Center for Clinical Science Research, Department of Radiation Oncology, Stanford University, Stanford, California (D.A.C., A.J.G.)

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: BiPS activates HIF.

Address correspondence to: Darren E. Richard, Centre de recherche de L'Hôtel-Dieu de Québec, 10 Rue McMahon, Québec QC G1R 2J6, Canada, E-mail: darren.richard@crhdq.ulaval.ca

Manuscript: 23 typed pages, 7 figures, 0 tables, 38 references

Word count: Abstract: 152 words

Introduction: 548 words

Discussion: 806 words

Abbreviations: HIF, Hypoxia-inducible factor; PHD, HIF prolyl hydroxylase; ODDD, oxygen dependant degradation domain; pVHL, von Hippel Lindau protein; FIH, Factor inhibiting HIF; MMP, matrix metalloprotease; BAEC, bovine aortic endothelial cells; VSMC, vascular smooth muscle cells; TFEIA, transcription factor enzyme immunoassay; 2-OG, 2-oxoglutarate.

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Hypoxia-inducible Factors (HIFs) are unstable heterodimeric transcription factors and decisive elements for the transcriptional regulation of genes important in the adaptation to low oxygen conditions. Hypoxia is the ubiquitous inducer of HIFs, stabilizing the α-subunit and permitting the formation of a functional HIF complex. Here, we identify (2R)-[(4-biphenylylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide (BiPS**)**, a commercially available metalloprotease -2 and -9 inhibitor, as a rapid and potent inducer of HIFs. We show that in different cell lines, BiPS induces the HIF-α subunit by inhibiting its degradation through stabilization of its labile oxygen-dependent degradation domain. This is achieved through the inhibition of HIF-1α hydroxylation. The HIF-1 complex, formed following BiPS treatment, is capable of DNA binding and activation of HIF target genes including the expression of vascular endothelial growth factor. Since novel HIF activators have generated considerable interest in the possible treatment of different ischemic diseases, we believe that BiPS and derivative molecules could have strong therapeutic potential.

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

Molecular oxygen is crucial for aerobic energy metabolism and cell survival. Hypoxia-inducible transcription factors (HIFs) are decisive elements in the adaptation to low oxygen conditions by permitting the expression of numerous genes involved in angiogenesis, anaerobic glycolysis and cell survival (Semenza, 2003). HIFs are heterodimers composed of an oxygen-sensitive subunit, HIF-1α, HIF-2α or HIF-3α and a constitutive HIF-β subunit. HIF-1α is ubiquitously expressed and has been highly characterized. The regulation of HIF-2α, also named HLF, EPAS1, HRF or MOP2, is similar to HIF-1α but its expression is more restricted (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997). HIF-3α has been less characterized and could act as a repressor of HIF-1 and HIF-2 activities (Gu et al., 1998; Makino et al., 2001; Makino et al., 2002).

Under normal oxygen conditions, the α subunit is hydroxylated through the action of HIF prolyl hydroxylases (PHDs). This hydroxylation occurs on two specific proline residues (Pro402 and Pro564 on human HIF-1α) contained in its oxygen-dependent degradation domain (ODDD). Hydroxylation of HIF-α allows the binding of pVHL, the product of the von Hippel-Lindau tumor suppressor gene. As the recognition component of an E3 ubiquitin ligase complex, pVHL allows for HIF-α polyubiquitination and subsequent proteosomal degradation (Schofield and Ratcliffe, 2004). Furthermore, HIF-α is hydroxylated on a specific asparagine residue (Asn803 on human HIF-1α) contained in its C-terminal transactivation domain by another dioxygenase, factor inhibiting HIF (FIH). Asn803 hydroxylation inhibits HIF transcriptional activity by preventing binding of the coactivator p300/CBP (Mahon et al., 2001). Inactivated by hypoxia, HIF-hydroxylases are dependent on oxygen and 2-oxoglutarate (2-OG) as substrates and ascorbate and iron as cofactors. Therefore, in most, if not all cell types, the lack of oxygen permits the stabilization of HIF-α and the formation of a transcriptionally active HIF complex.

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

Given the importance of HIF in response to ischemic conditions, the development of novel HIF inducers / activators has gained strong interest due to their therapeutic potential in the treatment of different ischemic disorders (Hewitson and Schofield, 2004; Khan et al., 2003; Simons and Ware, 2003) .

In this study, we present (2R)-[(4-biphenylylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide (BiPS**)** as a potent activator of both HIF-1 and HIF-2. This compound, derived from N-sulfonylamine acid, was originally designed as an inhibitor of matrix metalloproteases (MMP) -2 and -9 (Tamura et al., 1998). A number of studies using this compound have demonstrated potent effects in different *in vivo* and *in vitro* situations. These include: reducing lung colonization by Lewis lung carcinoma cells in mice (Tamura et al., 1998); blocking lymphocyte migration across endothelial cells (Deem and Cook-Mills, 2004); inhibiting of TGFβ-induced cataract formation in rat lens (Dwivedi et al., 2006); blocking the invasion of mouse brain microvessel endothelial cells in Matrigel (Fears et al., 2005); and impeding the migration of smooth muscle cells (Lin et al., 2007).

On top of its well characterized effects on MMP activity, here we show that BiPS can also induce HIF. In the present study, we demonstrate that BiPS stabilizes HIF-α protein by blocking HIF-α hydroxylation. This increase in HIF-α protein levels leads to the formation of active HIF complexes and the expression of HIF-target genes. Therefore, our study characterizes BiPS as a novel and potent activator of HIF complex, which could be of particular interest in the chemical pharmacology of the HIF signaling cascade.

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

Material and Methods

Materials. (2R)-[(4-biphenylylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide, known as MMP-2/MMP-9 Inhibitor II or BiPS (Tamura et al., 1998) was from Calbiochem / EMD Chemicals (Gibbstown, NJ). Cobalt chloride ($CoCl_2$), dimethyl-2-OG and MG132 were from Sigma (St. Louis, MO). pGL3 (R2.2) 3HRE-tk-LUC luciferase reporter vector was generated in our laboratory (Lauzier et al., 2007). GST-HIF-1α 344-582, pVHL-HA and CMV-Luc-ODD constructs were kindly provided by Drs Jacques Pouysségur (Institute of Signaling, Developmental Biology and Cancer Research, Université de Nice), Peter Ratcliffe (University of Oxford) and Richard K. Bruick (University of Texas), respectively.

Cell culture. VSMC were isolated from thoracic aorta of 6 week-old male Wistar rats (Owens et al., 1986) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (50 units/ml penicillin, 50 units/ml streptomycin). Bovine aortic endothelial cells (BAEC) were isolated from calf aortas and cultured in DMEM containing 10% FBS and antibiotics. HeLa cells were cultured in DMEM containing 10% heat-inactivated FBS and antibiotics. All cells were cultured in a humid atmosphere (5% CO_2, 95% air) and serially passaged upon reaching confluence. Hypoxic conditions were obtained by placing cells in a sealed hypoxic workstation (Ruskinn, Bridgend UK). The oxygen level in this workstation was maintained to 1% with the residual gas mixture containing 94% nitrogen and 5% carbon dioxide. All media for cell culture was from Invitrogen (Carlsbad, CA) unless otherwise indicated.

Western Blot analysis. Cells were lysed in 2× Leammli sample buffer. Protein concentration was determined by Lowry assay. Cell extracts (25 μg) were loaded on SDS-polyacrylamide gels

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

and transferred onto polyvinylidene difluoride (Immobilon-P, Millipore Corp, Billerica, MA) or nitrocellulose membranes (Hybond C Extra, GE Healthcare Life Sciences, Piscataway, NJ). Anti-HIF-1α and anti-HIF-2α antiserum were raised in rabbits immunized against the last 20 amino acids of the C termini of each human protein (Richard et al., 1999). Anti-hydroxylated HIF-1α against hydroxylated Pro402 and hydroxylated Pro564 of human sequence of HIF-1α were obtained as previously described (Chan et al., 2005). Monoclonal anti-HIF-1α and anti-GST antibodies and polyclonal anti-HIF-1β antibody were from Novus Biologicals (Littleton, CO). Monoclonal HA.11 antibody was from Convance (Emeryville, CA). Total polyclonal p42/p44 MAPK antibody was from Upstate (Lake Placid, NY) and used as a loading control. Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were from Promega (Madison, WI). Proteins were visualized with an enhanced chemiluminescence (ECL) system (GE Healthcare Life Sciences) or with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Western blots were quantified using Odyssey quantification software or ImageJ (http://rsb.info.nih.gov/ij).

RNA interference. To down-regulate HIF-α protein expression in HeLa cells, small interfering RNA (siRNA) duplexes targeting: human HIF-1α (accession no. NM_001530) sense: 5'-AGGACAAGUCACAACAGGAUU-3') and human HIF-2α (accession no. NM_001430) sense: 5'-GGGUCAGGUAGUAAGUGGCUU-3') were obtained from Ambion (Austin, TX). As a control, *Silencer*^{TM} *Negative Control #1 siRNA* was used (Ambion). HeLa cells were transfected with siRNA duplexes (20 nM) by calcium phosphate precipitation.

Luciferase assay. HeLa cells, seeded in 6 well plates, were transiently transfected by calcium phosphate precipitation with pGL3 (R2.2) 3HRE-tk-LUC luciferase reporter vector (1 μg/well) or

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

CMV-Luc-ODD (0.5 μg/well). *Renilla reniformis* luciferase expression vector (250 ng/well) was also used as a control for transfection efficiency. At 30 h post-transfection, cells were stimulated and luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Results were quantified with a Luminoskan Ascent microplate reader with integrated injectors (Thermo Electron, Milford, CA). Results are expressed as a ratio of firefly luciferase activity over *Renilla reniformis* luciferase activity. Experiments are an average ± SEM of triplicate data and representative of 3 independent experiments performed on different cell cultures.

Transcription factor enzyme-linked immunoassay. Experiments were performed as previously described (Blouin et al., 2004). Briefly, high-bind NeutrAvidin-coated 96-well strip plates (Pierce Biotechnology, Rockford, IL) were incubated with a 5'-biotinylated 26 base-pair dsDNA oligonucleotide for 1 h at room temperature. This sequence contains the previously described wild-type or mutant (underlined) HIF-1 binding motif (Semenza and Wang, 1992). Sequences used here were: 5'-GATCGCCCTACGTGCTGTCTCAGATC-3' for W26 wild-type sequence and 5'-GATCGCCCTAAAAGCTGTCTCAGATC-3' for M26 mutant sequence. Nuclear extracts from HeLa cells were incubated with oligonucleotides and HIF-1 complexes bound to DNA were detected using anti-HIF-1α or anti-HIF-β antibodies (Novus Biologicals), horseradish peroxidase-conjugated secondary antibodies and TMB-ONE (Promega). Experiments are an average ± SEM of triplicate data representative of 3 independent experiments performed on different cell cultures.

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

pVHL capture assay. HeLa cell were grown to confluence and stimulated as indicated. Cells were washed once in PBS and twice in HEB buffer (20 mM Tris pH7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol). Cells were then suspended and lysed using a Dounce homogenizer. Cytoplasmic extracts were cleared by centrifugation (20 000×g). Extracts (250 μg) were incubated with sepharose-bound GST-HIF-1α 344-582 (30 μg) for 1 h at room temperature, washed with NETN buffer (150 mM NaCl, 0.5 mM EDTA, 20 mM Tris pH 8.0, 0.5% Igepal, 100 μM deferoxamine) and incubated overnight with *in vitro* translated pVHL-HA in NETN at 4°C. Samples were washed with NETN, denatured in 2× Laemmli sample buffer, resolved in SDS-polyacrylamide gels (12%) and revealed by Western blotting with anti-HA and anti-GST antibodies.

Iron chelation assays. Iron chelation potential was determined using a ferrozine-based iron measurement assay (Fish, 1988; Stookey, 1970). Ferrous chloride ($FeCl_2$, 20 μM) was incubated with BiPS (75 μM) or deferoxamine (50 μM) for 30 minutes. Ferrozine (20 mg/ml) and ammonium acetate (1 mg/ml) was then added to the reaction mixture and incubated at 37°C for 30 minutes. Iron was measured by determining sample absorbance at 562 nm by spectrophotometry. The amount of free iron in the solution was determined by comparison with a $FeCl_2$/ferrozine standard curve. Experiments are an average ± SEM of triplicate data representative of 3 independent experiments.

Results

The effect of BiPS on HIF-1α and HIF-2α protein induction was evaluated in different cell lines including the established human HeLa cell line and primary cultures of bovine aortic

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

endothelial cells (BAEC) and rat aortic vascular smooth muscle cells (VSMC). As seen in Figure 1, BiPS induced HIF-α protein levels in all cell lines tested. In HeLa cells, a treatment with 10 μM BiPS for 2 hrs caused a detectable increase of both HIF-1α and HIF-2α proteins, while maximal induction was observed between 75 and 100 μM BiPS (Figure 1, upper panels). BiPS was more potent in BAEC since 5 μM led to a detectable increase of both HIF-α proteins, while maximal induction was observed at 25 μM (Figure 1, middle panels). In VSMC, 10 μM BiPS led to a detectable increase of HIF-1α protein, while maximal induction was observed at 50 μM BiPS (Figure 1, lower panels). However, we did not detect HIF-2α protein levels in VSMC treated with BiPS or under hypoxic conditions. HIF-1α and HIF-2α protein induction by BiPS was also observed in the macrophage-like cell line NR8383, human embryonic kidney cells (HEK-293), the murine endothelial cell line 1G11 and mouse embryonic fibroblasts (results not shown). It is interesting to note that the maximal induction of HIF-α following BiPS treatment was comparable to treatments under hypoxic conditions or in the presence of $CoCl_2$, two main inducers of HIF-α. Finally, other MMP-2/9 inhibitors, such as GM6001 (galardin) and MMP2/9 inhibitor I, did not induce HIF-α subunits (results not shown). These results indicate that the effect of BiPS on HIF-α induction was independent of MMP-2/9 inhibition and identify BiPS as a novel inducer of HIF-α subunits in normoxic cells.

The kinetics of HIF-α protein induction under BiPS treatment were rapid and comparable to hypoxic HIF-α induction. In HeLa cells, a detectable increase of HIF-1α was observed by 30 min and maximal induction was attained following 2 h of treatment with 75 μM BiPS (Figure 2). Interestingly, while HIF-1α levels were maintained in hypoxia for over 6 h, treatment of cells with BiPS maintained HIF-1α levels for up to 4 h and then subsequently decreased to basal

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

levels. HIF-2α protein induction by BiPS followed a similar kinetic pattern to HIF-1α. These results suggest that like hypoxia, BiPS stabilizes HIF-α instead of exploiting transcriptional or translational mechanisms for protein induction as seen for other normoxic inducers.

To directly evaluate the effect of BiPS on HIF-α subunit stabilization, HeLa cells were transfected with a construct encoding a luciferase protein chimera destabilized by the HIF-1α ODDD (Salnikow et al., 2004). In these conditions, changes in luciferase activity by treatment with BiPS would indicate the specific stabilization of HIF-1α's ODDD. As expected, the treatment of cells with $CoCl_2$ (Figure 3A) or the proteasome inhibitor MG132 (results not shown) strongly increased luciferase activity. More interestingly, treatment with BiPS also increased luciferase activity to similar levels as during MG132 and $CoCl_2$ treatments. This result indicates that BiPS stabilizes the HIF-1α ODDD.

Since decreased pVHL binding leads to increased HIF-α stabilization, we examined pVHL binding to HIF-1α following BiPS treatment. HeLa cells were treated with BiPS or $CoCl_2$ followed by the preparation of cytosolic extracts and *in vitro* hydroxylation of GST-HIF-1α (344-582). Following incubation with *in vitro* translated pVHL and GST pull-down, we observed that the treatment of cells with BiPS strongly prevented pVHL binding to HIF-1α (Figure 3B). Interestingly, the inhibition of pVHL binding with BiPS was more potent than with $CoCl_2$ treatment. This result suggests that BiPS inhibits PHD-mediated HIF-α hydroxylation. To confirm this, we evaluated the level of HIF-1α hydroxylation using two specific antibodies against hydroxylated proline residues Pro402 and Pro564 (Chan et al., 2005). For this experiment, cells were pretreated with MG132 and maintained in the presence of $CoCl_2$ or BiPS. As expected, the treatment of cells with $CoCl_2$ led to a near complete inhibition of HIF-1α protein hydroxylation of both proline residues (Figure 3C). More interestingly, treatment of cells

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

with BiPS also strongly decreased both HIF-1α Pro402 and Pro564 hydroxylation. Taken together, these results indicate that BiPS is a potent inhibitor of PHD activity.

We then wanted to investigate the mechanism by which BiPS inhibits PHD activity. Since PHD require Fe(II) to hydroxylate HIF-α subunits, we wanted to evaluate possibility that BiPS could inhibit PHD activity by acting as an iron chelating agent. In an iron chelation assay, deferoxamine, a well-known iron chelator and HIF-α stabilizing agent, was able to chelate over 80 % of the iron present in our experimental conditions (Figure 4A). However, BiPS did not act as an iron chelating agent since total free iron was completely accessible for reaction with ferrozine. This result indicates that BiPS does not inhibit PHD activity through iron chelation. PHDs belong to a large group of enzymes that use 2-oxoglutarate as a co-substrate (Schofield and Ratcliffe, 2004). We wanted to determine whether BiPS could interfere with the binding of 2-oxoglutarate to PHD. We therefore performed a pVHL capture assay in which 2-OG was added to the cellular extracts. In these conditions, the addition of 2-OG increased pVHL binding to HIF-1α (Figure 4B). More interestingly, the treatment of cellular extracts with BiPS blocked 2-OG-induced pVHL binding to HIF-1α. These results indicate that BiPS decreases PHD activity by interfering with 2-OG binding.

To determine the effect of BiPS treatment on HIF activity, we first evaluated its effect on the formation of an active HIF-1 transcription complex. To perform these studies, we used a HIF-1 transcription factor enzyme-linked immunoassay (TFEIA) (Blouin et al., 2004). Nuclear extracts from HeLa cells maintained in hypoxic conditions or in the presence of BiPS both demonstrated increased DNA-binding activity for HIF-1α and HIF-1β (Figure 5, W26). To control for specificity, we substituted the W26 dsDNA oligonucleotide sequence with a sequence mutated on 2 essential residues of the HIF-1-binding sequence (Figure 5, M26). In this case, very

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

little HIF-1 binding could be observed. This result indicates that BiPS stabilizes HIF-1α and permits the formation of the HIF-1 complex and binding to target HRE sequences.

We next attempted to determine whether BiPS treatment led to HIF-mediated transcriptional activity using a hypoxia response element (HRE)-controlled reporter assay. HeLa cells were transiently transfected with a 3HRE-tk-LUC reporter vector followed by treatment of cells with BiPS or in hypoxic conditions for 6 h. Under these conditions, hypoxia led to an 11 fold induction in luciferase activity (Figure 6). Interestingly, BiPS was more potent than hypoxia for activating HIF and increased reporter activity by 15.4 fold. We then studied the effect of BiPS treatment on the expression of a well-known HIF-1 activated target gene, vascular endothelial growth factor (Ebert et al., 1995; Forsythe et al., 1996; Liu et al., 1995; Mazure et al., 1996). As seen in Figure 7, the treatment of HeLa cells with BiPS strikingly increased VEGF expression. As in HRE reporter studies, BiPS was more potent than hypoxia for activating the expression of VEGF mRNA. To demonstrate the implication of HIF complexes in the increased expression of VEGF by BiPS, we used siRNA technology to deplete cells of HIF-1α and HIF-2α. The silencing of HIF-1α protein strongly reduced the expression of VEGF, while the silencing of HIF-2α caused a modest inhibition of the expression of this gene. Finally, the silencing of both HIF-α isoforms led to an additive inhibitory effect on VEGF mRNA expression. Effective silencing of HIF-1α and HIF-2α protein levels in HeLa cells is shown in the lower 2 panels of Figure 7. Taken together, our results identify BiPS as a potent activator of HIF complexes.

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

HIFs are decisive elements in the transcriptional regulation of genes implicated in cellular and physiological adaptation to hypoxic stress. The products of these genes are involved in several adaptive responses, including angiogenesis and erythropoiesis. A complex process, angiogenesis is regulated by several endogenous proteins and can be deregulated in tumor cell progression and during ocular and inflammatory pathologies. Angiogenesis may also be ineffective during ischemic stress, leading to tissue destruction. In this regard, the first therapeutic attempts to promote angiogenesis used the delivery of VEGF alone. This led to the production of leaky blood vessels and was proved to be ineffective in clinical trials (Ferrara, 2004). As a powerful regulator of many endogenous proangiogenic molecules, HIFs are considered appealing pharmacological targets for therapeutic angiogenesis. Different groups have shown the strong potential of HIF complexes in promoting vascularization in ischemic tissues (Elson et al., 2001; Li et al., 2000; Shyu et al., 2002; Vincent et al., 2000; Willam et al., 2002). Additionally, HIFs are strong regulators of erythropoiesis by inducing erythropoietin (Epo) production. A recombinant form of this specific hormone is commonly used for its ability to treat anemia. However, the high cost of this therapy, combined with the need for parenteral administration and development of anti-Epo antibodies has triggered the development of different approaches to stimulate erythropoiesis. Therefore, the discovery of novel HIF-inducing compounds should lead to new strategies to promote angiogenesis and treat anemia. Interestingly, specific PHD inhibitors have been shown to successfully increase the hemoglobin levels in rhesus macaques (Hsieh et al., 2007) and Epo levels in mice (Kasiganesan et al., 2007). Here, we have indentified BiPS, a MMP-2/9 inhibitor, as a novel and potent activator of HIFs. BiPS stabilizes HIF-α protein by blocking HIFα hydroxylation. This increase in HIF-α protein levels leads to the

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

formation of active HIF complexes and the expression of HIF-target genes. Therefore, BiPS or derivative molecules could have interesting therapeutic options towards HIF-regulated responses.

To our knowledge, the only compounds currently used to stabilize and activate HIFs are inhibitors of PHD activity. The hydroxylase activity of theses enzymes is modulated by their cofactors, namely O_2, 2-OG and Fe(II). The identification of PHD cofactors has led to the identification/design a of compounds able to increase HIFs, including compounds that chelate Fe(II) (deferoxamine) or compete with 2-OG binding to PHD (Hewitson and Schofield, 2004). Since BiPS can efficiently inhibit HIF-1α hydroxylation and pVHL binding without chelating iron, we believe that BiPS functions by interfering with the binding of 2-OG to PHD. This is supported by the demonstration that BiPS blocks the potentiation of pVHL binding by 2-OG. Additionally, using a docking simulation software (AutoDock; http://autodock.scripps.edu/), we performed a binding simulation of 2-OG and BiPS to PHD2 which corresponds to the current understanding of the 2-OG/PHD2 binding model (McDonough et al., 2006). In this simulation, the most probable position for BiPS binding to PHD2 was the 2-OG binding site, in the cavity containing the Fe(II) atom. The biphenyl group of BiPS was positioned inside the cavity while the 2 oxygen atoms of the sulfur group interacted with the Fe(II) atom. Furthermore, this model predicted that the PHD2 binding energy of BiPS is 1.65 times higher than 2-OG (mean of -7.009 kcal/mol ± 0.3 for BiPS and -4.245 kcal/mol ± 0.04 for 2-OG). Taken together, experimental and *in silico* evidence strongly suggests that BiPS induces and activates HIF by interfering with the binding of the PHD cofactor, 2-OG, leading to PHD inhibition.

We have successfully used BiPS for HIF induction in a variety of cell types. These characteristics reveal BiPS as an interesting compound for researchers interested in studying the HIF system. As a potent inhibitor of MMP2/9, BiPS was used by many different groups who were unaware of its effect on HIF activity. BiPS was utilized to study migration and invasion

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

properties of cells (Deem and Cook-Mills, 2004; Fears et al., 2005; Lin et al., 2007), to study the colonization of cancer cells in mice (Tamura et al., 1998) and to evaluate the remodeling and inflammation of airways during asthma (Lee et al., 2004). Depending on tissues targeted by BiPS the concentrations used, we believe that HIF activation could play an important role in the responses obtained using BiPS as a MMP2/9 inhibitor. We feel that researchers considering the use BiPS in their studies should strongly consider this possibility.

In this study, we present evidence that BiPS is a novel and potent PHD inhibitor in addition to its known role as a MMP2/9 inhibitor. A PHD inhibitor, BiPS prevents pVHL binding to HIF-α and its subsequent degradation. In addition, BiPS permits the transcriptional activity of HIFs and the expression of target genes. As PHD inhibitors are now recognized as potential therapeutic drugs in the treatment of anemia and ischemic diseases, we strongly believe that BiPS and derivative molecules could have strong therapeutic potential.

Acknowledgments

We are grateful to Guylaine Soucy for her technical assistance, Marc-André Déry for help with plasmid constructs, Éric Paquet for AutoDock expertise, Elisabeth Pagé and David Patten for helpful comments.

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

References

Blouin CC, Page EL, Soucy GM and Richard DE (2004) Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1alpha. *Blood* **103**(3):1124-1130.

Chan DA, Sutphin PD, Yen SE and Giaccia AJ (2005) Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha. *Mol Cell Biol* **25**(15):6415-6426.

Deem TL and Cook-Mills JM (2004) Vascular cell adhesion molecule 1 (VCAM-1) activation of endothelial cell matrix metalloproteinases: role of reactive oxygen species. *Blood* **104**(8):2385-2393.

Dwivedi DJ, Pino G, Banh A, Nathu Z, Howchin D, Margetts P, Sivak JG and West-Mays JA (2006) Matrix metalloproteinase inhibitors suppress transforming growth factor-beta-induced subcapsular cataract formation. *Am J Pathol* **168**(1):69-79.

Ebert BL, Firth JD and Ratcliffe PJ (1995) Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. *J Biol Chem* **270**(49):29083-29089.

Elson DA, Thurston G, Huang LE, Ginzinger DG, McDonald DM, Johnson RS and Arbeit JM (2001) Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1alpha. *Genes Dev* **15**(19):2520-2532.

Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y and Fujii-Kuriyama Y (1997) A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc Natl Acad Sci U S A* **94**(9):4273-4278.

Fears CY, Grammer JR, Stewart JE, Jr., Annis DS, Mosher DF, Bornstein P and Gladson CL (2005) Low-density lipoprotein receptor-related protein contributes to the antiangiogenic activity of thrombospondin-2 in a murine glioma model. *Cancer Res* **65**(20):9338-9346.

Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* **25**(4):581-611.

Fish WW (1988) Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. *Methods Enzymol* **158**:357-364.

Flamme I, Frohlich T, von Reutern M, Kappel A, Damert A and Risau W (1997) HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. *Mech Dev* **63**(1):51-60.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* **16**(9):4604-4613.

Gu YZ, Moran SM, Hogenesch JB, Wartman L and Bradfield CA (1998) Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expr* **7**(3):205-213.

Hewitson KS and Schofield CJ (2004) The HIF pathway as a therapeutic target. *Drug Discov Today* **9**(16):704-711.

Hogenesch JB, Chan WK, Jackiw VH, Brown RC, Gu YZ, Pray-Grant M, Perdew GH and Bradfield CA (1997) Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J Biol Chem* **272**(13):8581-8593.

Hsieh MM, Linde NS, Wynter A, Metzger M, Wong C, Langsetmo I, Lin A, Smith R, Rodgers GP, Donahue RE, Klaus SJ and Tisdale JF (2007) HIF prolyl hydroxylase inhibition results in endogenous erythropoietin induction, erythrocytosis, and modest fetal hemoglobin expression in rhesus macaques. *Blood* **110**(6):2140-2147.

Kasiganesan H, Sridharan V and Wright G (2007) Prolyl hydroxylase inhibitor treatment confers whole-animal hypoxia tolerance. *Acta Physiol (Oxf)* **190**(2):163-169.

Khan TA, Sellke FW and Laham RJ (2003) Gene therapy progress and prospects: therapeutic angiogenesis for limb and myocardial ischemia. *Gene Ther* **10**(4):285-291.

Lauzier MC, Page 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**(8):4023-4031.

Lee KS, Jin SM, Kim SS and Lee YC (2004) Doxycycline reduces airway inflammation and hyperresponsiveness in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol* **113**(5):902-909.

Li J, Post M, Volk R, Gao Y, Li M, Metais C, Sato K, Tsai J, Aird W, Rosenberg RD, Hampton TG, Sellke F, Carmeliet P and Simons M (2000) PR39, a peptide regulator of angiogenesis. *Nat Med* **6**(1):49-55.

Lin SJ, Lee IT, Chen YH, Lin FY, Sheu LM, Ku HH, Shiao MS, Chen JW and Chen YL (2007) Salvianolic acid B attenuates MMP-2 and MMP-9 expression in vivo in apolipoprotein-E-deficient mouse aorta and in vitro in LPS-treated human aortic smooth muscle cells. *J Cell Biochem* **100**(2):372-384.

Liu Y, Cox SR, Morita T and Kourembanas S (1995) Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circ Res* **77**(3):638-643.

Mahon PC, Hirota K and Semenza GL (2001) FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* **15**(20):2675-2686.

Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A and Poellinger L (2001) Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature* **414**(6863):550-554.

Makino Y, Kanopka A, Wilson WJ, Tanaka H and Poellinger L (2002) Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. *J Biol Chem* **277**(36):32405-32408.

Mazure NM, Chen EY, Yeh P, Laderoute KR and Giaccia AJ (1996) Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. *Cancer Res* **56**(15):3436-3440.

McDonough MA, Li V, Flashman E, Chowdhury R, Mohr C, Lienard BM, Zondlo J, Oldham NJ, Clifton IJ, Lewis J, McNeill LA, Kurzeja RJ, Hewitson KS, Yang E, Jordan S, Syed RS and Schofield CJ (2006) Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proc Natl Acad Sci U S A* **103**(26):9814-9819.

Owens GK, Loeb A, Gordon D and Thompson MM (1986) Expression of smooth muscle-specific alpha-isoactin in cultured vascular smooth muscle cells: relationship between growth and cytodifferentiation. *J Cell Biol* **102**(2):343-352.

Richard DE, Berra E, Gothie E, Roux D and Pouyssegur J (1999) p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. *J Biol Chem* **274**(46):32631-32637.

Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM and Kasprzak KS (2004) Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. *J Biol Chem* **279**(39):40337-40344.

Schofield CJ and Ratcliffe PJ (2004) Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* **5**(5):343-354.

Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**(10):721-732.

Semenza GL and Wang GL (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* **12**(12):5447-5454.

Stookey L (1970) Ferrozine-A New Spectrophotometric Reagent for Iron. *Anal Chem* **42**(7):779-781.

Tamura Y, Watanabe F, Nakatani T, Yasui K, Fuji M, Komurasaki T, Tsuzuki H, Maekawa R, Yoshioka T, Kawada K, Sugita K and Ohtani M (1998) Highly selective and orally active

inhibitors of type IV collagenase (MMP-9 and MMP-2): N-sulfonylamino acid derivatives. *J Med Chem* **41**(4):640-649.

Tian H, McKnight SL and Russell DW (1997) Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* **11**(1):72-82.

Vincent KA, Shyu KG, Luo Y, Magner M, Tio RA, Jiang C, Goldberg MA, Akita GY, Gregory RJ and Isner JM (2000) Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1alpha/VP16 hybrid transcription factor. *Circulation* **102**(18):2255-2261.

Willam C, Masson N, Tian YM, Mahmood SA, Wilson MI, Bicknell R, Eckardt KU, Maxwell PH, Ratcliffe PJ and Pugh CW (2002) Peptide blockade of HIFalpha degradation modulates cellular metabolism and angiogenesis. *Proc Natl Acad Sci U S A* **99**(16):10423-10428.

Footnote

This work was supported by grants from the Canadian Institutes of Health Research (CIHR, MOP-49609) and the Heart and Stroke Foundations of Québec and Canada. D.E.R. is a recipient of a CIHR New Investigator Award. M.C.L. is a recipient of a Graduate Scholarship from the CIHR.

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Figure 1 : HIF-α induction by BiPS.

Quiescent VSMC, quiescent BAEC or HeLa cells were maintained under control conditions, in hypoxic conditions (1% O_2), in the presence of $CoCl_2$ (200 μM) or various concentrations of BiPS or vehicle (DMSO 0.08%) for 2 h. Total cell extracts (25 μg) were resolved by SDS-PAGE (8%) and immunoblotted using anti-HIF-1α, anti-HIF-2α or anti-p42/p44 MAPK antibodies.

Figure 2 : Time-course for HIF-α induction by BiPS.

HeLa cells were maintained under control conditions, in hypoxic conditions (1% O_2) or in the presence of 75 μM of BiPS or vehicle (DMSO 0.08%) for different periods of time. Total cell extracts (25 μg) were resolved by SDS-PAGE (8%) and immunoblotted using anti-HIF-1α, anti-HIF-2α or anti-p42/p44 MAPK antibodies.

Figure 3 : Stabilization of HIF-1α by BiPS .

A) HeLa cells were transfected with 0.5 μg of CMV-luc-HIF-1α-ODDD and 250 ng of an expression vector coding for Renilla reniformis luciferase. At 40 h post-transfection, cells were maintained under control conditions, hypoxic conditions (1% O_2) or in the presence of BiPS (75 μM) or MG132 (5 μM) for 6 h. Cells were lysed and luciferase activity was measured. Results are expressed as a ratio of beetle luciferase activity to *R. reniformis* luciferase activity and are an average ± SEM of at least 3 independent experiments performed in triplicate. B) Cytoplasmic extracts from HeLa cells treated as indicated were incubated with HIF-1α (344-582) GST protein-coupled sepharose beads. Samples were then incubated overnight in the presence of *in*

vitro translated pVHL and resolved by SDS-PAGE (12%). Immunoblotting was performed using anti-HA (pVHL) and anti-GST antibodies. C) HeLA cells were treated with MG132 (25 μM) for 2 h and followed by the addition of $CoCl_2$ (200 μM) or BiPS (75 μM) for an additional 2 h. Total cell extracts (25 μg) were resolved by SDS-PAGE (8%) and immunoblotted using anti-hydroxylated-Pro402 (P402-OH), anti-hydroxylated-Pro564 (P564-OH), anti-HIF-1α or anti-p42/p44 MAPK antibodies.

Figure 4 : Modulation of PHD activity by BiPS

A) $FeCl_2$ (20 μM) was incubated with BiPS (75 μM) or deferoxamine (50 μM) for 30 minutes. Ferrozine (20 mg/ml) and ammonium acetate (1 mg/ml) was then added to the reaction mixture and incubated at 37^0C for 30 minutes. Results are expressed as total free iron as determined by comparison with a $FeCl_2$/ferrozine standard curve and are an average ± SEM of 3 independent experiments. B) Cytoplasmic extracts from HeLa cells were supplemented with dimethyl-2-oxoglutarate (33 μM) and/or treated with BiPS (100 μM) for 20 minutes prior to incubation with HIF-1α (344-582) GST protein-coupled sepharose beads. Samples were then incubated overnight in the presence of *in vitro* translated pVHL and resolved by SDS-PAGE (12%). Immunoblotting was performed using anti-HA (pVHL) and anti-GST antibodies.

Figure 5 : Increased HIF-1 nuclear complex formation by BiPS.

HeLa cells were maintained under control conditions, in hypoxic conditions (1% O_2) or in the presence of BiPS (75 μM) or vehicle (DMSO 0.08%) for 2 h. Nuclear protein extract (10 μg) was incubated in a 96-well plate coated with an oligonucleotide containing the wild-type (W26) or mutant (M26) HIF-1-binding site. The presence of HIF-1 transcription complex was evaluated

Molecular Pharmacology Fast Forward. Published on April 18, 2008 as DOI: 10.1124/mol.108.045690
This article has not been copyedited and formatted. The final version may differ from this version.

using anti-HIF-1α and anti-HIF-1β antibodies. Results are expressed as the fold increase of absorbance at 450 nm over control conditions and are an average ± SEM of at least 3 independent experiments performed in triplicate.

Figure 6 : HIF-dependent reporter gene activation by BiPS.

HeLa cells (6-well plate) were transfected with 1 μg of a pGL3 (R2.2) 3HRE-TK reporter construct and 250 ng of an expression vector coding for *Renilla reniformis* luciferase to normalize transfection efficiency. At 40 h post-transfection, cells were maintained under control conditions, in hypoxic conditions (1% O_2) or in the presence of BiPS (75 μM) or vehicle (DMSO 0.08%) for 6 h. Cells were lysed and luciferase activity was measured. Results are expressed as a ratio of beetle luciferase activity to *R. reniformis* luciferase activity and are an average ± SEM of at least 3 independent experiments performed in triplicate.

Figure 7 : HIF-dependent VEGF expression by BiPS.

BAEC were transfected with 20 nM of siRNA oligonucleotides targeting HIF-1α, HIF-2 or with a control siRNA. At 40 h post-transfection, cells were maintained under control conditions, in hypoxic conditions (1% O_2) or in the presence of BiPS (75 μM) or vehicle (DMSO 0.08%) for 2 h. Upper 2 panels: Total RNA was extracted and resolved on formaldehyde/agarose gels. Northern blot was performed using a specific radiolabeled VEGF probe. An 18S RNA probe was used as a control for gel loading. Lower 2 panels: Total cell extracts (25 μg) were resolved on SDS-PAGE (8%) and immunoblotted using anti-HIF-1α and anti-HIF-2α antibodies.

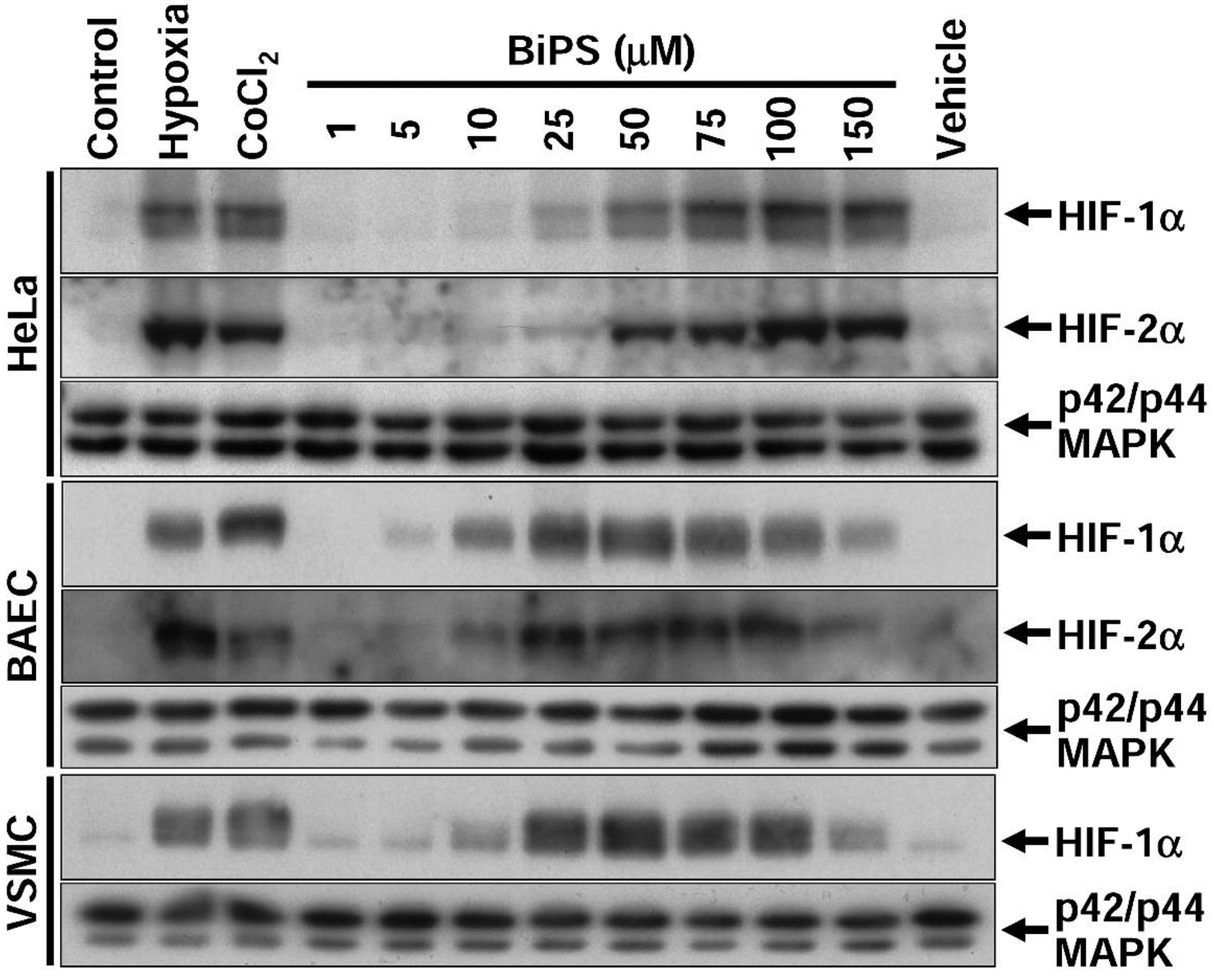
Control
Hypoxia
CoCl2
BiPS (μM)
1
5
10
25
50
75
100
150
Vehicle
HeLa
BAEC
VSMC
HIF-1α
HIF-2α
p42/p44 MAPK
HIF-1α
HIF-2α
p42/p44 MAPK
HIF-1α
p42/p44 MAPK

Figure 1

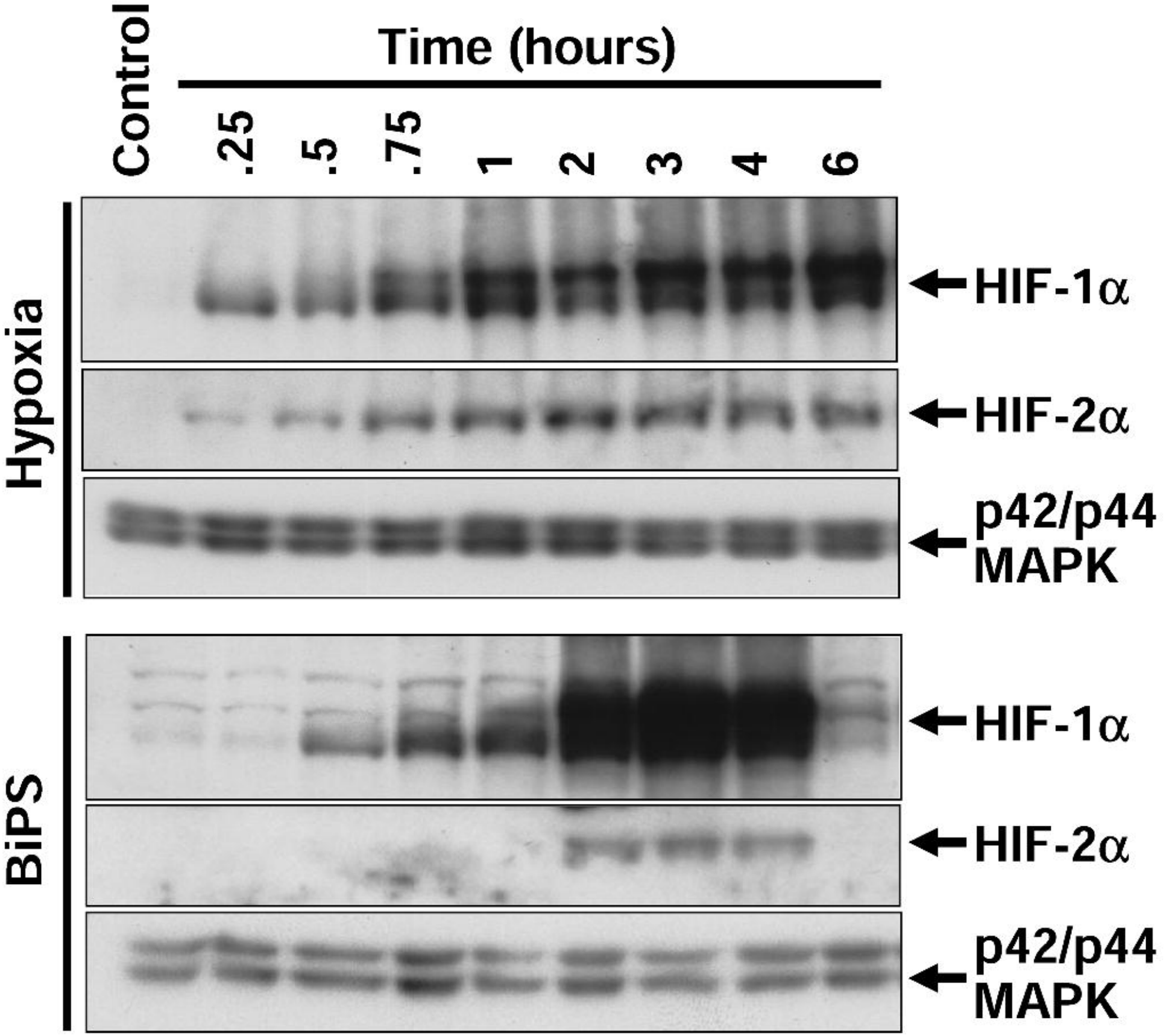
Control
Time (hours)
.25
.5
.75
1
2
3
4
6
Hypoxia
HIF-1α
HIF-2α
p42/p44 MAPK
BiPS
HIF-1α
HIF-2α
p42/p44 MAPK

Figure 2

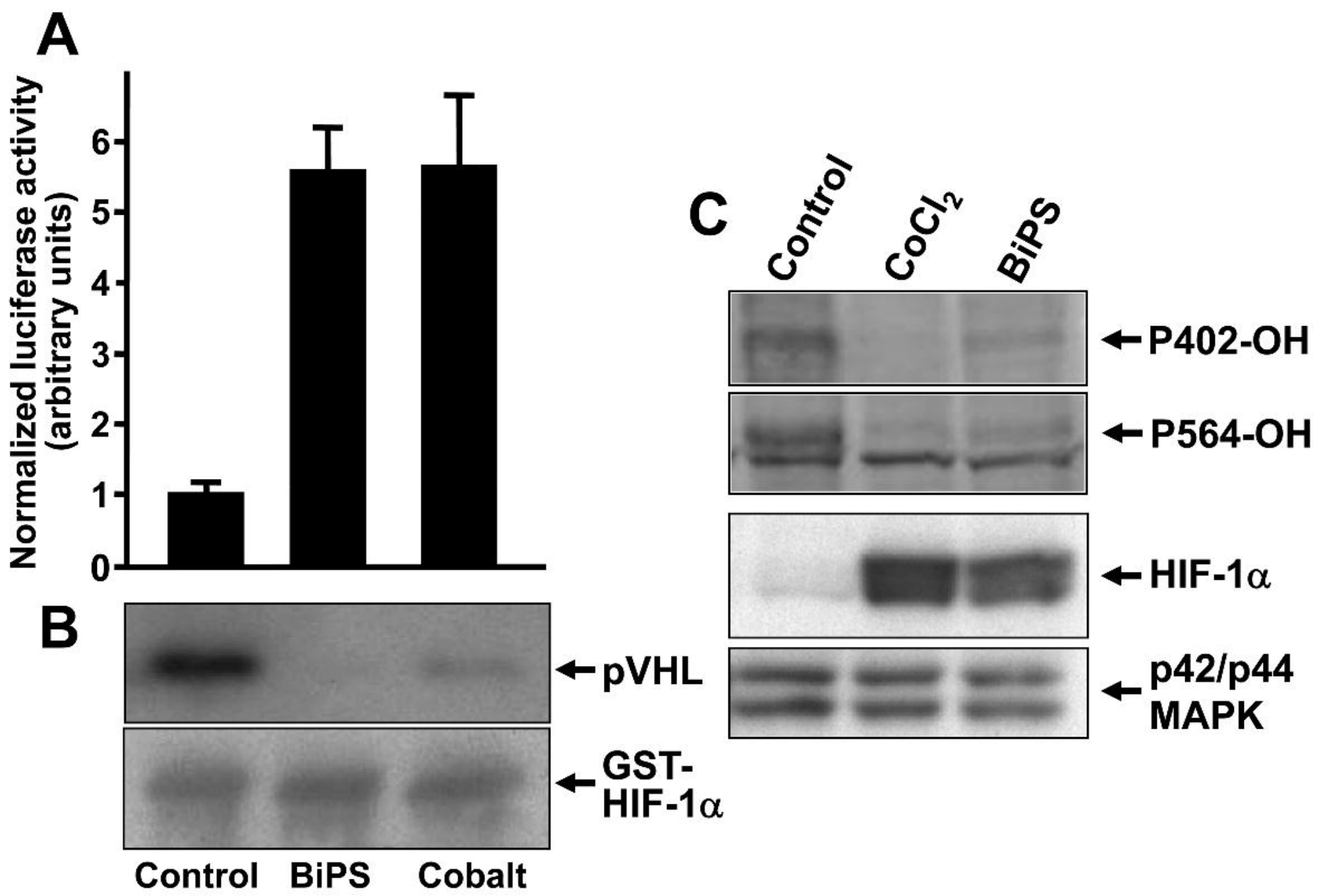
A
Normalized luciferase activity (arbitrary units)
0
1
2
3
4
5
6
B
pVHL
GST-HIF-1α
Control
BiPS
Cobalt
C
Control
CoCl2
BiPS
P402-OH
P564-OH
HIF-1α
p42/p44 MAPK

Figure 3

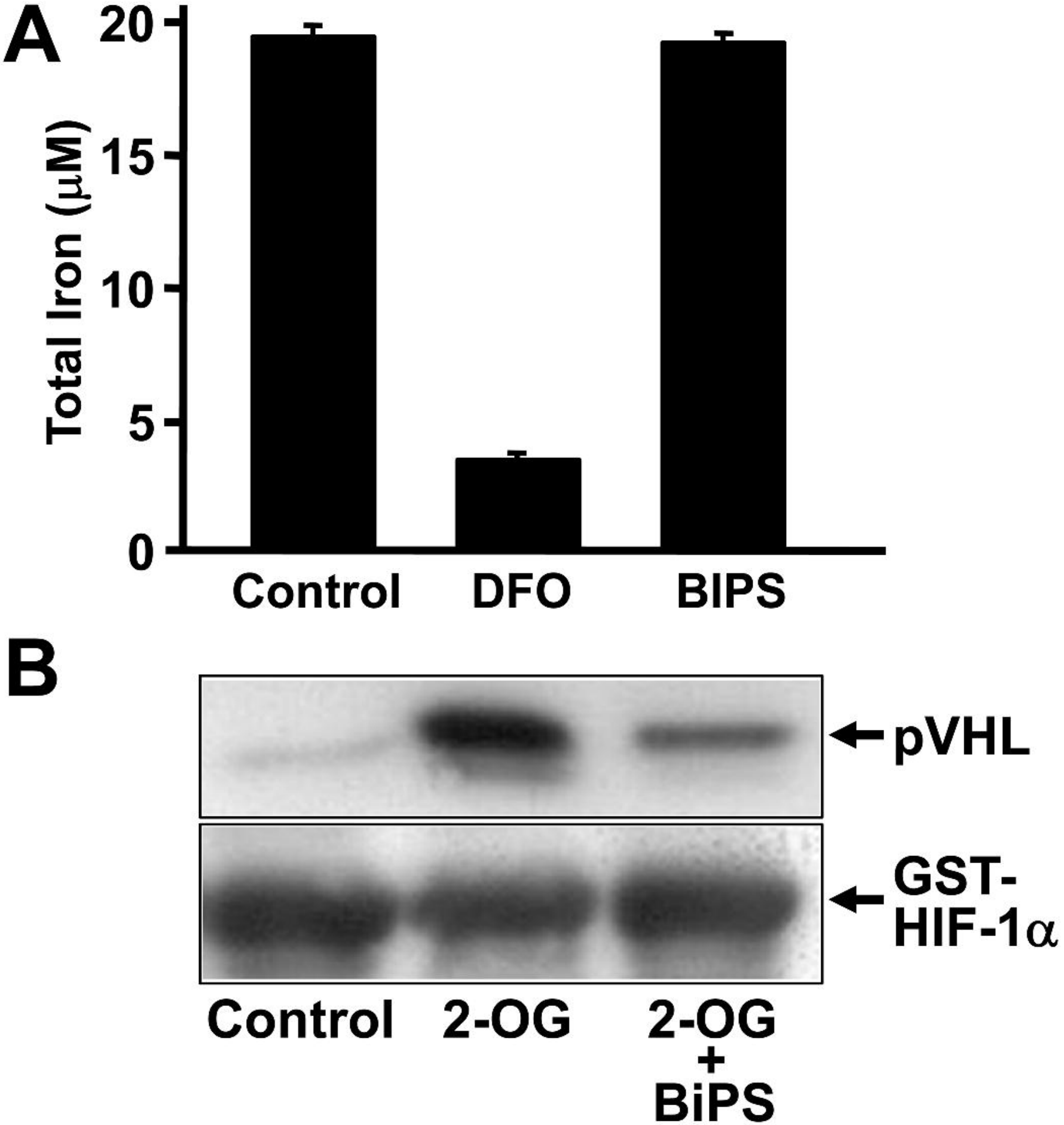
A
Total Iron (μM)
20
15
10
5
0
Control
DFO
BIPS
B
pVHL
GST-
HIF-1α
Control
2-OG
2-OG
+
BiPS

Figure 4

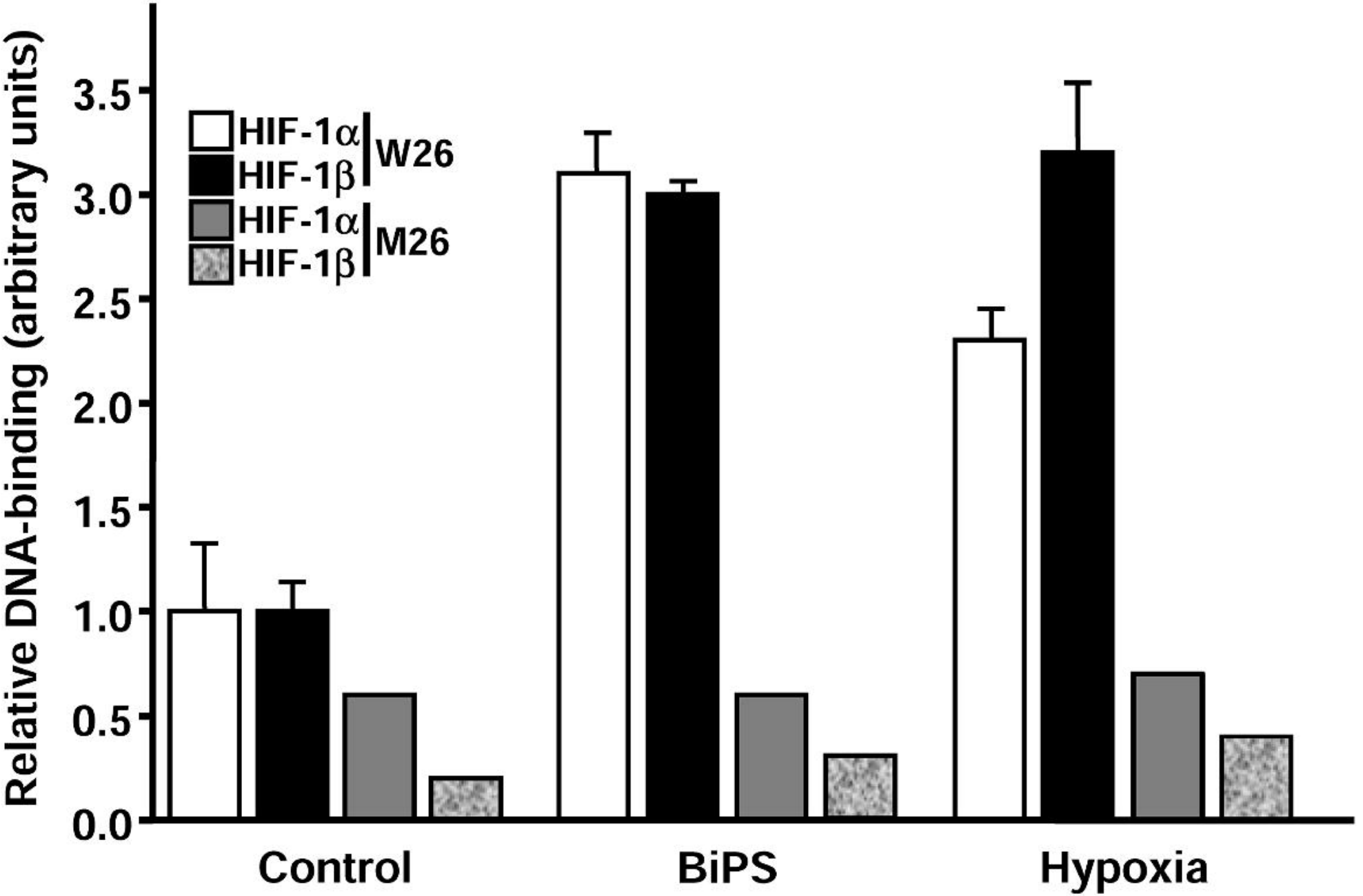
Relative DNA-binding (arbitrary units)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
HIF-1α
HIF-1β
W26
HIF-1α
HIF-1β
M26
Control
BiPS
Hypoxia

Figure 5

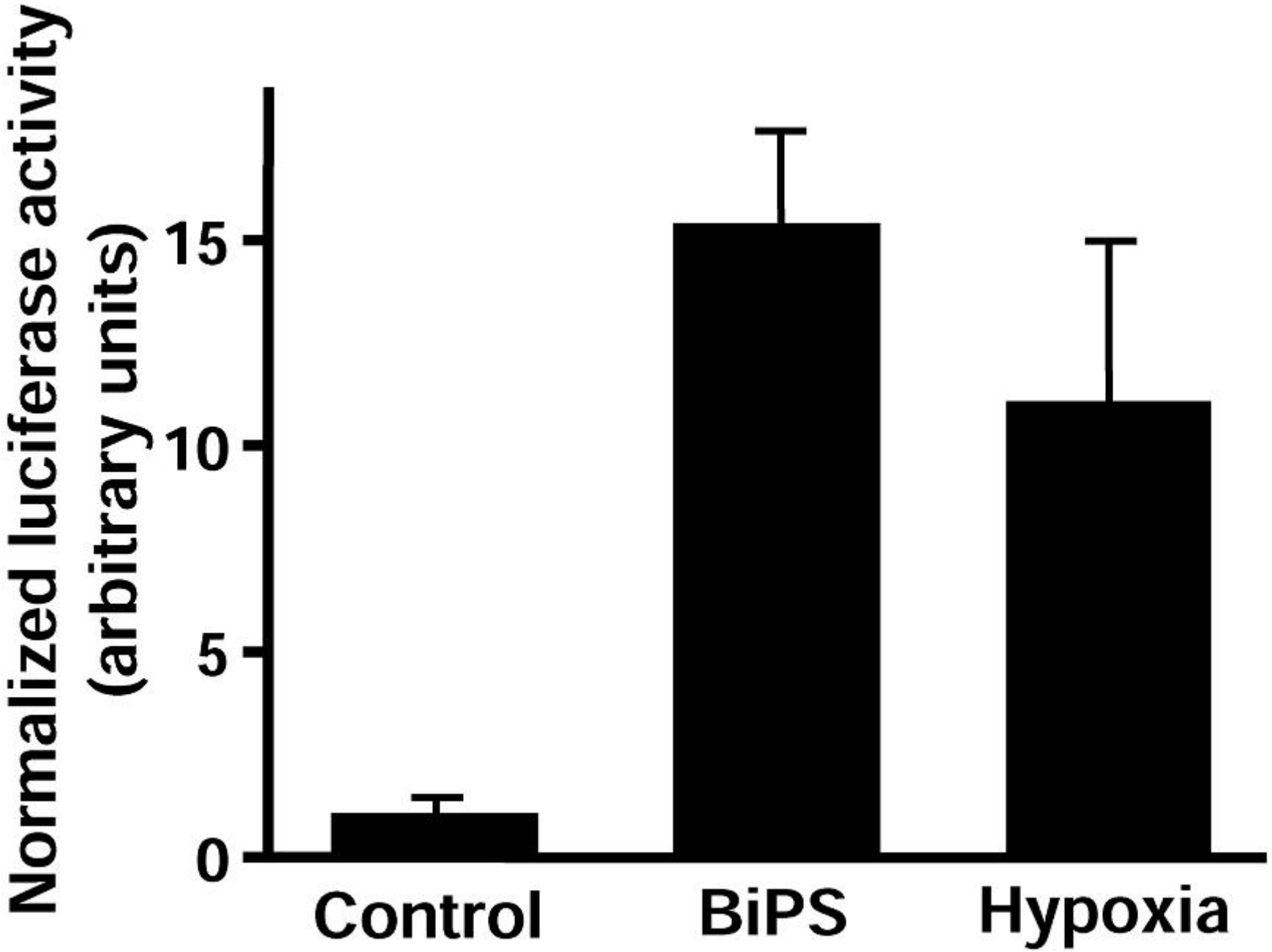
Normalized luciferase activity
(arbitrary units)
15
10
5
0
Control
BiPS
Hypoxia

Figure 6

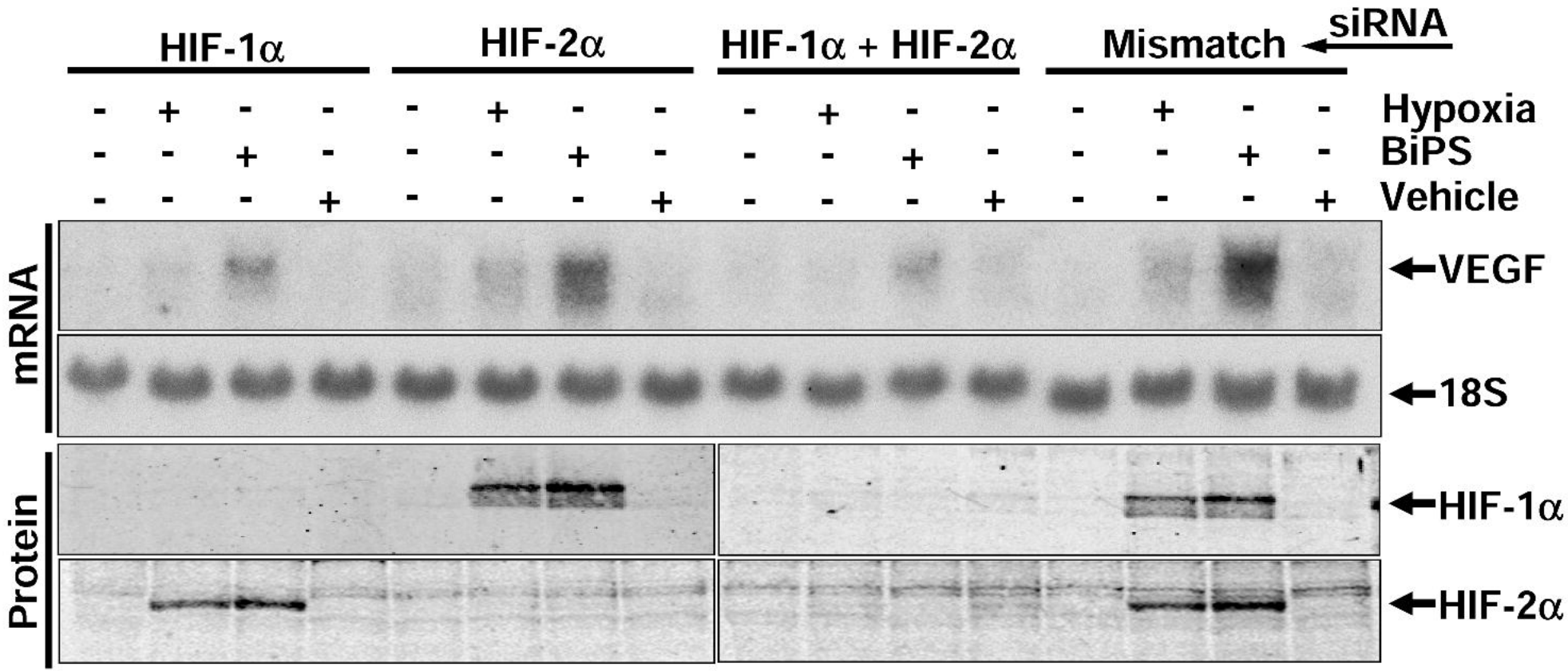
HIF-1α
HIF-2α
HIF-1α + HIF-2α
Mismatch
siRNA
Hypoxia
BiPS
Vehicle
mRNA
VEGF
18S
Protein
HIF-1α
HIF-2α

Figure 7