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Hypoxia-inducible Factor-1α Stabilization in Nonhypoxic Conditions: Role of Oxidation and Intracellular Ascorbate Depletion

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Hypoxia-inducible factor-1 (HIF-1) is a decisive element for the transcriptional regulation of many genes induced under low oxygen conditions. Under normal oxygen conditions, HIF-1α, the active subunit of HIF-1, is hydroxylated on proline residues by specific HIF prolyl-hydroxylases, leading to ubiquitination and degradation by the proteasome. In hypoxia, hydroxylation and ubiquitination are blocked and HIF-1α accumulates in cells. Recent studies have shown that in normal oxygen conditions G-protein–coupled receptor agonists, including angiotensin (Ang) II and thrombin, potently induce and activate HIF-1 in vascular smooth muscle cells. The current study identifies HIF-1α protein stabilization as a key mechanism for HIF-1 induction by Ang II. We show that hydroxylation on proline 402 is altered by Ang II, decreasing pVHL binding to HIF-1α and allowing HIF-1α protein to escape subsequent ubiquitination and degradation mechanisms. We show that HIF-1α stability is mediated through the Ang II–mediated generation of hydrogen peroxide and a subsequent decrease in ascorbate levels, leading to decreased HIF prolyl-hydroxylase activity and HIF-1α stabilization. These findings identify novel and intricate signaling mechanisms involved in HIF-1 complex activation and will lead to the elucidation of the importance of HIF-1 in different Ang II–related cell responses.

INTRODUCTION

Oxygen is an essential element in the biology of every aerobic organism. Tissue and cellular regulation of oxygen supply is essential to mediate adaptation mechanisms during low oxygen conditions. At the cellular level, the hypoxia-inducible transcription factor, HIF-1, is a key regulator of responses in low oxygen conditions. HIF-1 specifically binds hypoxic response element (HRE)-driven promoters on a number of genes that include vascular endothelial growth factor (VEGF), heme oxygenase, glucose transporter-1, and erythropoietin (Semenza *et al.*, 1991; Semenza and Wang, 1992; Lee *et al.*, 1997). HIF-1 is a heterodimeric complex composed of HIF-1α and HIF-1β. HIF-1β is found in all cells, whereas HIF-1α is the oxygen-regulated subunit (Wang *et al.*, 1995).

HIF-1α is highly unstable and the mechanisms controlling HIF-1α degradation in normoxic conditions have been well described (Schofield and Ratcliffe, 2005). Human HIF-1α is hydroxylated on two proline residues; Pro^{402} and Pro^{564}. Both residues are situated in the oxygen-dependent degradation domain (ODDD) of HIF-1α. Three different HIF prolyl-hydroxylases have been described, termed PHD1, PHD2, and PHD3 for their prolyl hydroxylase domain (Bruick and McKnight, 2001; Epstein *et al.*, 2001). PHD enzymes hydroxylate HIF-1α using oxygen and 2-oxoglutarate as substrates and iron and ascorbate as essential cofactors. Although all three PHDs have been shown to regulate HIF-1α, the key isoform responsible for HIF-1α regulation in many cell types is PHD2 (Berra *et al.*, 2003; Appelhoff *et al.*, 2004). It has been shown that PHD1 and PHD3 also hydroxylate HIF-1α in vivo and in vitro (Epstein *et al.*, 2001; Appelhoff *et al.*, 2004). However, the exact relevance of these two isoforms in cells remains to be elucidated.

HIF-1α hydroxylation allows the binding of pVHL, the product of the von Hippel-Lindau tumor suppressor gene. pVHL is the recognition component of a E3 ligase complex necessary for ubiquitination and subsequent proteasome-dependent degradation of HIF-1α (Huang *et al.*, 1998; Maxwell *et al.*, 1999; Cockman *et al.*, 2000; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). In hypoxic conditions, low oxygen leads to HIF-1α stabilization due to the inhibition of proline hydroxylation and subsequent decreases in HIF-1α ubiquitination and degradation. HIF-1α is stabilized and forms, in combination with the HIF-1β subunit, the active HIF-1 complex.

HIF-1 regulation by nonhypoxic stimuli has gained considerable interest (Feldser *et al.*, 1999; Hellwig-Burgel *et al.*, 1999; Richard *et al.*, 2000; Gorlach *et al.*, 2001; Laughner *et al.*,

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Abbreviations used: Ang II, angiotensin II; HIF, hypoxia-inducible factor; HRE, hypoxic response element; GPCR, G-protein–coupled receptor; ODDD, oxygen-dependent degradation domain; PHD, HIF prolyl-hydroxylases; pVHL, von Hippel-Lindau protein; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; VSMCs, vascular smooth muscle cells.

2001). In vascular smooth muscle cells (VSMCs), angiotensin (Ang) II and thrombin are strong inducers of HIF-1α and potent activators of HIF-1 (Richard *et al.*, 2000; Gorlach *et al.*, 2001; Page *et al.*, 2002; Lauzier *et al.*, 2007). Interestingly, these two stimuli induce HIF-1α in VSMCs through markedly different pathways than hypoxic induction (Page *et al.*, 2002; BelAiba *et al.*, 2004; Bonello *et al.*, 2007; Lauzier *et al.*, 2007). Ang II activates at least two separate pathways to induce HIF-1α protein levels in VSMCs under normoxic conditions. First, activation of diacylglycerol-sensitive protein kinase C (PKC) plays an important part in increasing the transcription of HIF-1α. Second, Ang II increases HIF-1α translation by a reactive oxygen species (ROS)-dependent activation of the phosphatidylinositol 3-kinase (PI3K)/p70S6 kinase (p70S6K) pathway and through the 5′-untranslated region (5′-UTR) of HIF-1α mRNA (Page *et al.*, 2002). However, in these normoxic conditions, the HIF-1α protein degradation system should be at its full capacity. This is not the case because the induction and activation of HIF-1α during Ang II treatment is fully comparable to that seen under hypoxic conditions (Richard *et al.*, 2000). Therefore, the goal of present study was to investigate the possibility that the treatment of VSMCs with Ang II may also regulate HIF-1α protein stabilization.

Here, we demonstrate that HIF-1α stabilization after Ang II treatment is similar to that seen in hypoxic conditions. We show that the stimulation of VSMCs with Ang II stabilizes HIF-1α through its ODDD and regulates HIF-1α hydroxylation, leading to changes in HIF-1α ubiquitination and proteasome targeting. Our studies indicate that Ang II regulates PHD enzyme activity through the modulation of intracellular ascorbate concentrations, reducing the availability of this cofactor. Finally, we show that Ang II–induced hydrogen peroxide (H_2O_2) production participates in decreasing intracellular ascorbate levels and promotes HIF-1α stability. These results identify intricate signaling mechanisms involved in HIF-1α protein regulation after the activation of VSMCs through G-protein–coupled receptors.

MATERIALS AND METHODS

Materials

Ang II, thrombin, ascorbate, H_2O_2 and catalase were from Sigma (St. Louis, MO). MG132 was from Calbiochem (La Jolla, CA). GST-S5a-agarose was from BIOMOL (Plymouth Meeting, PA). Easytag express protein labeling mix was from Perkin Elmer (Boston, MA). Anti-HIF-1 antiserum was raised by our laboratory in rabbits immunized against the last 20 amino acid of the C termini of human HIF-1α (Richard *et al.*, 1999). Anti-hydroxylated HIF-1α against hydroxylated Pro^{402} and hydroxylated Pro^{564} of the human sequence of HIF-1α were obtained as previously described (Chan *et al.*, 2002, 2005). Monoclonal anti-phospho-p42/p44 MAPK, anti-tubulin, and the monoclonal anti-β-actin antibodies were from Sigma. Total polyclonal p42/p44 MAPK antibody was from Upstate (Lake Placid, NY). Anti-phospho-p70S6K (Thr^{389}) antibody was from Cell Signaling (Beverly, MA). Anti-GST antibody was from Novus Biologicals (Littleton, CO). Monoclonal HA.11 antibody was from Convance (Emeryville, CA). Anti-ubiquitin was from Boston Biochem (Cambridge, MA). Horseradish peroxidase–coupled anti-mouse and anti-rabbit antibodies were from Promega (Madison, WI). Glutathione *S*-transferase (GST)-HIF-1α, pVHL-hemagglutinin (HA), and luc-HIF-1α-ODDD 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

VSMCs were isolated from the thoracic aorta of 6-wk-old male Wistar rats by enzymatic dissociation (Owens *et al.*, 1986). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 U/ml streptomycin (Invitrogen, Carlsbad, CA) in a humid atmosphere (5% CO_2, 95% air). Cells were passaged upon reaching confluence, and all experiments were performed between passages 4 and 12. Cells were serum-deprived for 16 h before treatment. Hypoxic conditions were obtained by placing cells in a sealed hypoxic workstation (Ruskinn, Bridgend, United Kingdom). The oxygen level in this workstation was maintained at 1%, with the residual gas mixture containing 94% nitrogen and 5% carbon dioxide.

Western Blot Analysis

Confluent cells were lysed in 2× Laemmli sample buffer. Protein concentration was determined by Lowry assay. Samples were resolved in SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Proteins were revealed with specific antibodies as indicated and visualized with an enhanced chemiluminescence (ECL) system (GE Healthcare Life Sciences, Piscataway, NJ) or with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Western blots were quantified using Odyssey quantification software or Scion Image (Frederick, MD; http://www.scioncorp.com).

Northern Blot Analysis

Confluent cells were lysed and RNA was isolated with TRIzol reagent (Invitrogen). RNA resolved on 1% agarose/6% formaldehyde gels was transferred to Hybond N+ nylon membranes (GE Healthcare Life Sciences) and hybridized with a radioactive cDNA probe against the total coding sequence of the mouse VEGF gene. A probe against 18S rRNA was used as a loading control.

Luciferase Assay

VSMCs, seeded in six-well plates, were transfected with the cytomegalovirus (CMV)-luc-HIF-1α-ODDD luciferase reporter vector (1 μg/well). *Renilla reniformis* luciferase expression vector (250 ng/well) was also used as a control for transfection efficiency. Transfection was performed on 45% confluent cells with Superfect transfection reagent (Qiagen, Valencia, CA) at a 1:3 DNA/reagent ratio. Six hours after transfection, fresh medium was added to cells. Forty-eight hours after transfection, cells were deprived of FBS for 16 h and stimulated as indicated for 6 h. Cells were washed with cold phosphate-buffered saline, and luciferase assays were performed with 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 to *Renilla reniformis* luciferase activity. Experiments are an average ± SEM of triplicate data representative of three independent experiments performed on different cell cultures.

Proteasome-binding Assay

VSMCs were grown to confluence, serum-deprived for 16 h, and stimulated as indicated for 4 h. Cells were then washed with phosphate-buffered saline (PBS) and lysed in lysis buffer [20 mM Tris, pH 7.5, 5% glycerol, 0.1% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)]. Lysates were centrifuged (20,000 × *g*) for 15 min at 4°C. VSMC extracts (equal amounts of HIF-1α protein) were incubated with 10 μg of GST-S5a-agarose for 2 h at 4°C in S5a buffer (20 mM Tris, 140 mM NaCl, 5% glycerol, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM AEBSF). Samples were washed with S5a buffer containing 0,1% Triton and denatured in 2× Laemmli sample buffer. Samples were resolved in SDS-polyacrylamide gels (6%) and revealed by Western blot analysis.

In Vitro Ubiquitination Assay

VSMCs were grown to confluence, serum-deprived for 16 h, and stimulated as indicated for 4 h. Cells were washed once in PBS and twice in HEB buffer (20 mM Tris, pH 7.5, 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*). ^{35}S-radiolabeled HIF-1α, translated in vitro using the TnT Coupled Reticulocyte Lysate System (Promega), was incubated for the times indicated with 100 μg of cytoplasmic extracts in 0.5 mg/ml ubiquitin, 15 μM ubiquitin aldehyde, 2 mM Tris, pH 7.5, 1 mM ATP, 1 mM magnesium acetate, 30 mM phosphocreatine, and 0.05 mg/ml creatine phosphokinase. Samples were denatured in 2× Laemmli sample buffer and resolved in SDS-polyacrylamide gels (6%). Gels were dried and exposed to Kodak BioMax MS film (Eastman Kodak, Rochester, NY).

pVHL Capture Assay

VSMCs were grown to confluence, serum-deprived for 16 h and stimulated as indicated for 4 h. Cytoplasmic extracts (250 μg), prepared as described above, were incubated with Sepharose-bound GST-HIF-1α (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 desferioxamine) 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.

Determination of Intracellular Ascorbate Concentrations

VSMCs were grown to confluence in 100-mm plates in DMEM supplemented with ascorbate (asDMEM, 250 μM). Cells were serum-deprived for 16 h, and fresh asDMEM was added for 1 h before stimulation for 4 h in DMEM. Cells were then washed in PBS, harvested, centrifuged, and resuspended in a 90% methanol/1 mM EDTA solution. Samples were vigorously vortexed and centrifuged at 20,000 $\times$ *g*. Ascorbate levels were analyzed by spectrophotometry as previously described (Queval and Noctor, 2007). Briefly, samples were mixed with 0.1 mM NaH_2PO_4, pH 5.6, and absorbance at 265 nm was measured by spectrophotometry. Ascorbate peroxidase (0.4 U) was then added to the samples for 3 min, and absorbance at 265 nm was again measured. Ascorbate concentrations were determined as the difference in absorbance prior and after the addition of ascorbate peroxidase. Alternatively, ascorbate levels were measured by HPLC as previously described (Levine *et al.*, 2001). A Lowry protein assay was used for normalization of the samples.

RESULTS

Ang II Regulates HIF-1α Protein Degradation

Past work showed that Ang II induced HIF-1α in VSMCs through transcriptional and translational mechanisms (Page *et al.*, 2002). Given the elevated levels of HIF-1α protein induction during Ang II treatment and the high instability of HIF-1α under normal oxygenation, we undertook studies to determine if Ang II treatment stabilized HIF-1α protein levels in VSMCs. The half-life of HIF-1α protein was evaluated using cycloheximide, a general protein synthesis inhibitor. VSMCs, stimulated with Ang II or hypoxia for 4 h, were treated with cycloheximide to block all novel HIF-1α protein synthesis. Half-life of HIF-1α was then evaluated by Western blotting. As seen in Figure 1, the half-life of HIF-1α protein in Ang II–treated cells is similar to the half-life of HIF-1α protein under hypoxic conditions. These results indicate that Ang II stimulation of VSMCs causes the stabilization of HIF-1α protein levels.

In normoxic conditions, HIF-1α is rapidly ubiquitinated and targeted for proteosomal degradation. We attempted to determine whether Ang II could affect ubiquitination and proteosomal targeting. To address this, we studied the HIF-1α ubiquitination potential of VSMC extracts. These experiments were performed by incubating cytoplasmic VSMC extracts with ^{35}S-radiolabeled in vitro–translated HIF-1α protein. Ubiquitinated HIF-1α was then resolved on SDS-polyacrylamide gels. As shown in Figure 2A, the appearance of high-molecular bands denotes the ubiquitination state of HIF-1α. Interestingly, cell extracts from Ang II–treated cells have a weaker capacity to ubiquitinate HIF-1α than unstimulated VSMCs. This is particularly evidenced by the weaker intensity of high-molecular bands at all time points during the assay. This result suggests that HIF-1α ubiquitination is decreased in VSMCs after Ang II treatment. To demonstrate the effect of Ang II on endogenous HIF-1α ubiquitination, we performed GST pulldown assays using a GST-S5a fusion protein. The S5a protein is a non-ATPase subunit of the 26S proteasome that binds polyubiquitinated proteins with a high affinity and its utilization for the purification of polyubiquitinated proteins has been well described (Layfield *et al.*, 2001; Groulx and Lee, 2002; Heessen *et al.*, 2002). Total VSMC extracts (equal amounts of HIF-1α protein) were incubated with GST-S5a protein cou-

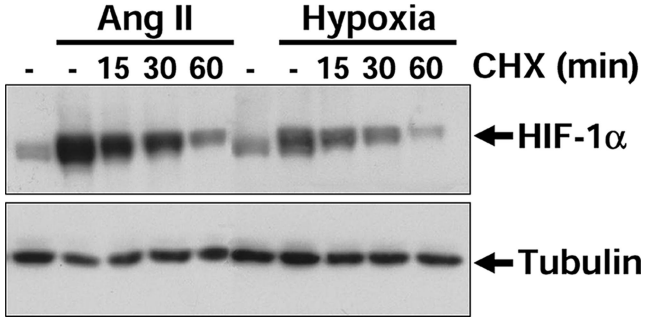

Figure 1. Ang II modifies HIF-1α protein half-life. Quiescent VSMCs were maintained under control conditions, in the presence of Ang II (100 nM) or in hypoxic conditions (1% O_2) for 4 h. Cycloheximide (CHX, 30 μg/ml) was then added for the indicated times. Total cell extracts (25 μg) were resolved by SDS-PAGE (8%) and immunoblotted using anti-HIF-1α and anti-tubulin antibodies.

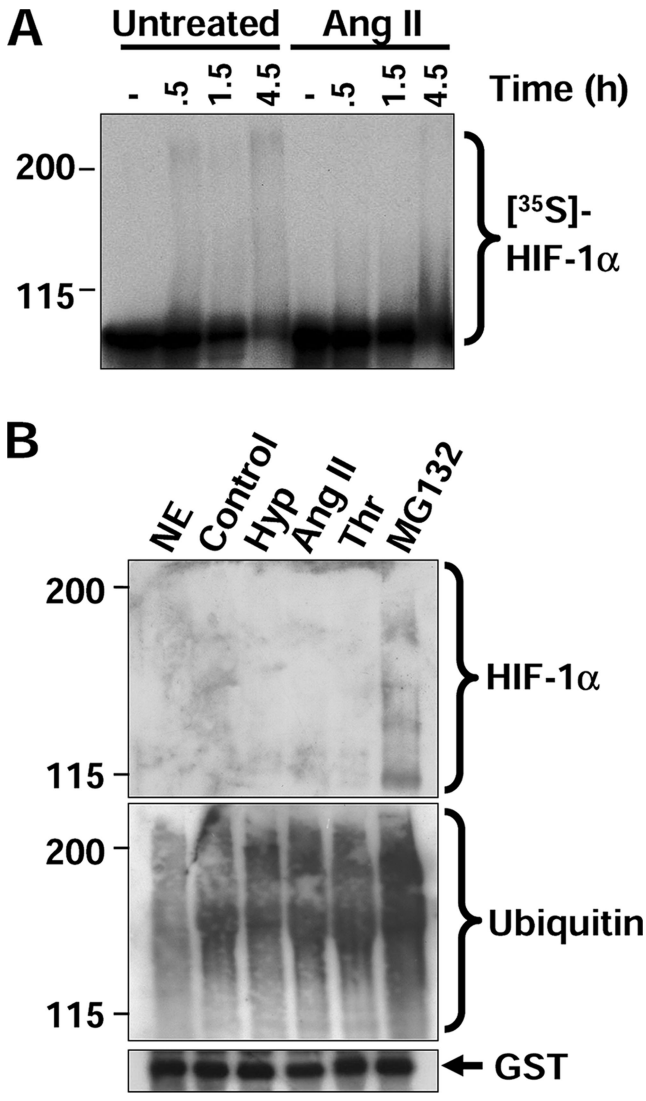

Figure 2. Ang II modifies HIF-1α protein ubiquitination. (A) Quiescent VSMCs were maintained under control conditions or in the presence of Ang II (100 nM) for 4 h. Cytoplasmic extracts were prepared and incubated with in vitro–translated and ^{35}S-radiolabeled HIF-1α for in vitro ubiquitination (see *Materials and Methods*). Samples were then resolved by SDS-PAGE (6%). Gels were dried and exposed for ^{35}S autoradiography. (B) Total cell extracts (1 mg) maintained in control conditions, in hypoxic conditions (Hyp, 1% O_2), or in the presence of Ang II (100 nM), thrombin (Thr, 5 U/ml), or MG132 (20 μM) for 4 h were incubated with 10 μg GST-S5a-agarose protein for 2 h. Alternatively, GST-S5a-agarose protein was incubated without cell extracts (NE). Samples were resolved by SDS-PAGE (6%) and immunoblotted with anti-HIF-1α, anti-ubiquitin, and anti-GST antibodies.

pled to agarose beads. The level of bound HIF-1α was then evaluated by Western blotting. As seen in Figure 2B, extracts treated with the proteasome inhibitor MG132 demonstrated increased HIF-1α protein binding to S5a. As expected, when cells were treated in hypoxic conditions, HIF-1α binding to S5a was blocked, a hallmark of decreased HIF-1α ubiquitination in these conditions. More interestingly, VSMCs treated with Ang II also blocked HIF-1α binding to S5a. Taken together, these results show that HIF-1α ubiquitination is decreased after the treatment of VSMCs with Ang II and suggest that HIF-1α stabilization is the result of an impairment of HIF-1α ubiquitination. It is important to note that thrombin, another nonhypoxic activator of HIF-1 (Richard *et al.*, 2000; Gorlach *et al.*, 2001), also blocks HIF-1α proteasome binding and ubiquitination (Figure 2B and Supplementary Figure S1) through pathways similar to those that will be described below for Ang II.

Ang II Regulates HIF-1α Protein Hydroxylation

We next attempted to determine the effect of Ang II on upstream signaling events leading to HIF-1α ubiquitination. As mentioned previously, HIF-1α ubiquitination and degradation are controlled through its ODDD. To determine whether Ang II could control the stability of the ODDD, we utilized a fusion protein construct comprised of amino acids 401-602 of the HIF-1α ODDD and the C-terminal end of the firefly luciferase protein. This construct generates an unstable form of luciferase when transfected into cells. The half-life of this luciferase construct is increased by oxygen deprivation and can be quantified by traditional luciferase assays (Salnikow *et al.*, 2004). VSMCs were transiently transfected with the CMV-luc-HIF-1α-ODDD vector. As expected, treatment of cells with MG132 or hypoxia increased luciferase activity in VSMCs by 3.3- and 2.0-fold over basal levels, respectively (Figure 3). More interestingly, the stimulation of VSMCs with Ang II also increased luciferase activity by 1.9-fold. This result demonstrates that Ang II targets the HIF-1α ODDD to increase HIF-1α protein stabilization.

HIF-1α ubiquitination and degradation are controlled by the hydroxylation of two specific proline residues (Pro^{402} and Pro^{564} for human HIF-1α) contained in the ODDD. PHD2 is the prolyl-hydroxylase shown to be the key regulator of HIF-1α protein levels in many cell types (Berra *et al.*, 2003). The expression of PHD2 has been shown to be decreased by TGF-β1, a nonhypoxic inducer of HIF-1α

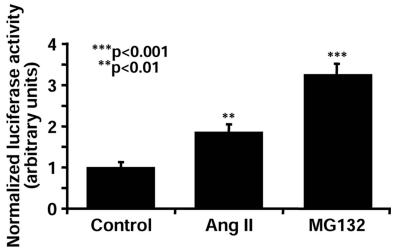

Figure 3. Ang II regulates the HIF-1α ODDD. VSMCs were transfected with 1 μg of CMV-luc-HIF-1α-ODDD encoding a luc-HIF-1α-ODDD fusion protein and 250 ng of an expression vector encoding *Renilla reniformis* luciferase. Six hours after transfection, cells were serum-deprived for 16 h. Cells were maintained under control conditions or stimulated with Ang II (100 nM) or MG132 (20 μM) for 6 h. VSMCs were lysed and luciferase activity was measured using the Dual-Luciferase reporter assay. Results are expressed as a ratio of beetle luciferase activity to *R. reniformis* luciferase activity and are an average ± SEM of at least three independent experiments performed in triplicate.

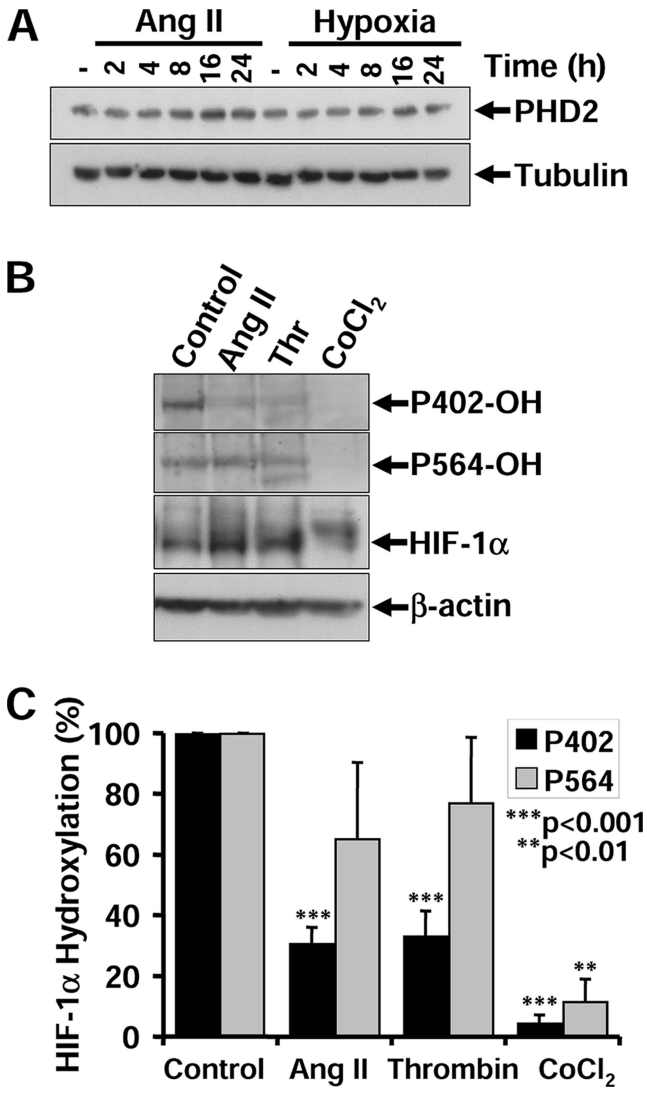

Figure 4. Ang II regulates HIF-1α protein hydroxylation. (A) Quiescent VSMCs were maintained under control conditions, in the presence of Ang II (100 nM) or in hypoxic conditions (1% O_2) for indicated times. Total cell extracts (25 μg) were resolved by SDS-PAGE (10%) and immunoblotted using anti-PHD2 and anti-tubulin antibodies. (B) Quiescent VSMCs were treated with MG132 (20 μM) and stimulated with Ang II (100 nM), thrombin (thr, 5 U/ml) or $CoCl_2$ (200 μM) for 4 h. Total cell extracts (25 μg) were resolved by SDS-PAGE (8%) and immunoblotted using anti-hydroxylated-Pro^{402} (P402-OH), anti-hydroxylated-Pro^{564} (P564-OH), anti-HIF-1α, and anti-β-actin antibodies. (C) Western blots in B were quantified with Scion Image or the Odyssey Infrared Imaging System using β-actin as an internal loading control. Results are expressed as a percentage of hydroxylated HIF-1α (normalized to total HIF-1α protein levels) on Pro^{402} (black bars) or Pro^{564} (gray bars) compared with untreated cells (control) and are an average ± SD of at least three independent experiments.

(McMahon *et al.*, 2006). However, in VSMCs we did not observe any decrease in PHD2 protein expression after Ang II treatment (Figure 4A), demonstrating that HIF-1α protein stabilization by Ang II is not a consequence of decreased PHD2 expression.

To investigate the possibility that the treatment with Ang II could regulate hydroxylase activity, we evaluated the level of HIF-1α hydroxylation using two specific antibodies

against hydroxylated proline residues Pro^{402} and Pro^{564} (Chan *et al.*, 2005). For this assay, cells were treated with MG132 and maintained with or without Ang II, thrombin, or $CoCl_2$, a hypoxia mimetic that strongly inhibits HIF-1α hydroxylation. As expected, the treatment of VSMCs with $CoCl_2$ led to a near complete inhibition of HIF-1α protein hydroxylation of both proline residues (Figure 4B). Interestingly, treatment of VSMCs with Ang II also significantly decreased HIF-1α protein hydroxylation. The effect of Ang II was predominantly on Pro^{402} hydroxylation, reducing this modification by 69.2 ± 5.2% compared with control cells treated only with MG132 (Figure 4C). Alternatively, we could detect no significant effect of Ang II treatment on HIF-1α Pro^{564} hydroxylation. Finally, the treatment of cells with thrombin also blocked HIF-1α hydroxylation in a manner similar to Ang II (Figure 4, B and C).

A key step in HIF-1α protein degradation is its binding to pVHL, which is a direct consequence of HIF-1α hydroxylation. To determine the effect of Ang II treatment on pVHL binding, a pVHL capture assay was used. A GST-HIF-1α fusion protein, comprised of amino acids 344-582 from human HIF-1α, was subjected to modification by Ang II–treated cell extracts, followed by interaction with in vitro–translated pVHL protein. As seen in the left panel of Figure 5A, the treatment of cells with Ang II decreased pVHL binding to HIF-1α. As expected, the treatment of VSMCs with $CoCl_2$ completely abolished pVHL binding to HIF-1α. To control the specificity of pVHL binding to hydroxylated HIF-1α, a mutated form of the GST-HIF-1α fusion protein (proline residues Pro^{402} and Pro^{564} to alanine) was used. Using this mutated construct, no pVHL binding was observed (right panel of Figure 5A). Furthermore, time-course experiments indicated that the treatment of VSMCs with Ang II decreased pVHL binding to HIF-1α between 2 and 4 h of stimulation (Figure 5B). This time course is similar to previous studies on HIF-1α induction, which also showed a maximal HIF-1α protein induction between 2 and 4 h of Ang II treatment (Page *et al.*, 2002). Taken together, our results demonstrate that Ang II modulates hydroxylation of HIF-1α on Pro^{402}, leading to decreased pVHL binding and increased HIF-1α stability.

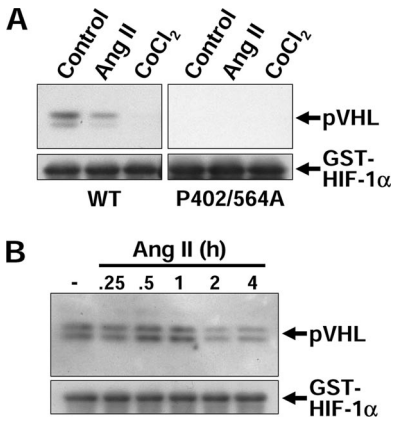

Figure 5. Ang II regulates pVHL binding to HIF-1α. (A and B) VSMCs were stimulated as indicated and cytoplasmic extracts were incubated with wild-type or mutated (P402/564A) GST-HIF-1α protein coupled to Sepharose beads for 1 h. Samples were then incubated overnight in the presence of in vitro–translated pVHL and resolved by SDS-PAGE (12%). Immunoblotting was performed using anti-HA (pVHL) and anti-GST antibodies.

Ang II Modifies Cellular Levels of Ascorbate, a PHD Cofactor

As mentioned previously, PHDs belong to the family of 2-oxoglutarate–dependent dioxygenases and catalyze the ferrous iron (Fe^{2+})-dependent hydroxylation of HIF-1α. Moreover, PHDs are also dependent on the presence of ascorbate for maximal activity (Kivirikko and Myllyharju, 1998; Knowles *et al.*, 2003). Recent studies have demonstrated that ascorbate levels can be regulated by certain stimuli shown to induce HIF-1α (Salnikow *et al.*, 2004; Kaczmarek *et al.*, 2007). Therefore, we attempted to evaluate the possibility that Ang II treatment could regulate the activity of PHD through the modulation of intracellular ascorbate levels. We first set out to determine if ascorbate supplementation could modify HIF-1α induction by Ang II treatment. We found that ascorbate supplementation in culture medium completely blocked HIF-1α protein induction in VSMCs after Ang II treatment (Figure 6A). Interestingly, ascorbate supplementation also decreased the expression of VEGF, a HIF-1 target gene, after Ang II treatment (Figure 6C). Ascorbate supplementation did not affect other signaling pathways activated through the AT_1 receptor, such as p42/p44 MAPK or the downstream target of the PI3K pathway, p70S6K. Ascorbate supplementation also did not affect the hypoxic induction of HIF-1α (Figure 6B). Finally, ascorbate supplementation restored the ability of pVHL to bind

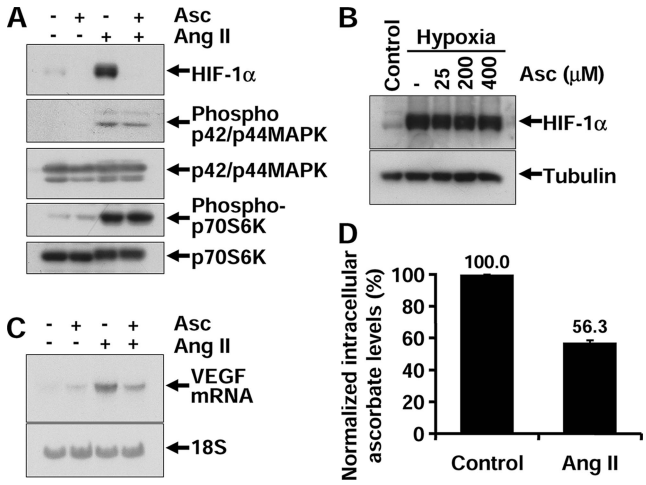

Figure 6. Impact of intracellular ascorbate on HIF-1α stabilization. (A and B) Quiescent VSMCs were pretreated or not with ascorbate (Asc, 5 μM or indicated concentrations) for 15 min and maintained under control conditions, in the presence of Ang II (100 nM) or in hypoxic conditions for 4 h. Total cell extracts (25 μg) were resolved by SDS-PAGE (8%) and immunoblotted using anti-HIF-1α, anti-phospho-p42/p44 MAPK, anti-p42/p44 MAPK, anti-phospho-p70S6K, anti-p70S6K, and anti-tubulin antibodies. (C) Quiescent VSMCs were pretreated or not with 5 μM ascorbate for 15 min and maintained under control conditions or in the presence of Ang II (100 nM) for 4 h. Total RNA was extracted and resolved in formaldehyde/agarose gels. Northern blots were performed using a specific radiolabeled VEGF probe. An 18S RNA probe was used as a control for gel loading. (D) Quiescent VSMCs were incubated in ascorbate-supplemented DMEM for 16 h. Cells were then maintained under control conditions or in the presence of Ang II (100 nM) in nonsupplemented DMEM. Cells were washed with PBS and resuspended in 90% methanol/1 mM EDTA solution. Ascorbate concentrations were measured in samples using HPLC (see *Materials and Methods*). Results are expressed as the percentage of intracellular ascorbate concentrations compared with untreated cells and are an average ± SEM of at least three independent experiments performed in triplicate.

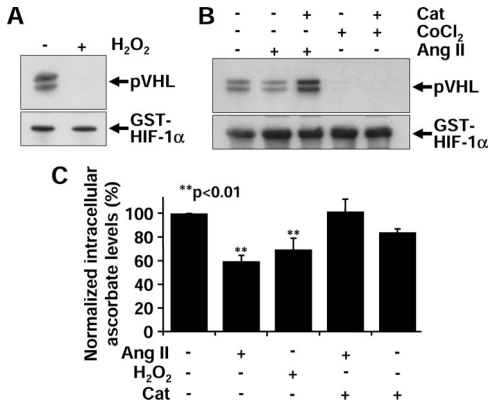

Figure 7. Hydrogen peroxide is responsible for decreased HIF-1α hydroxylation and ascorbate depletion during Ang II treatment. (A) Extracts from quiescent VSMCs were treated with or without H_2O_2 (100 μM). (B) Quiescent VSMCs were pretreated with or without catalase (cat, 1000 U/ml) and maintained under control conditions, in the presence of Ang II (100 nM) or $CoCl_2$ (200 μM). Cytoplasmic extracts were incubated with GST-HIF-1α protein coupled to 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) Quiescent VSMCs were incubated in ascorbate-supplemented DMEM for 16 h. Cells were maintained under control conditions or in the presence of H_2O_2 (50 μM), catalase (1000 U/ml) and/or Ang II (100 nM) in nonsupplemented DMEM. Cells were washed with PBS and resuspended in 90% methanol/1 mM EDTA solution. Ascorbate concentrations were measured in samples using spectrophotometry (see *Materials and Methods*). Results are expressed as the percentage of intracellular ascorbate concentrations compared with untreated cells and are an average ± SEM of at least three independent experiments performed in triplicate.

HIF-1α (results not shown). Taken together, these results indicate that the supplementation of VMSC with ascorbate restores the ability of the PHD enzymes to hydroxylate HIF during Ang II treatment and inhibits Ang II–induced HIF-1α protein stability.

Recent studies have demonstrated that cobalt and nickel ions regulate intracellular ascorbate levels leading to HIF-1α protein induction (Salnikow *et al.*, 2004). Moreover, it has been shown that the depletion of intracellular ascorbate levels leads to HIF-1α protein up-regulation (Vissers and Wilkie, 2007). Therefore, we determined whether Ang II treatment could modify intracellular ascorbate levels in VMSC. Using both HPLC (Figure 6D) and spectrophotometric (Figure 7C) determinations of intracellular ascorbate levels, our results demonstrated that the treatment of VSMCs with Ang II decreased intracellular levels of ascorbic acid by 56.3 ± 2.4% compared with nontreated cells. These results suggest that decreased ascorbate availability in VSMCs during Ang II stimulation leads to increased HIF-1α protein stability.

It is well accepted that Ang II activates the production of reactive oxygen species in VSMCs (Griendling *et al.*, 1994; Kimura *et al.*, 2005; Lyle and Griendling, 2006; Zhang *et al.*, 2007). The major ROS products generated by Ang II in VSMCs are hydroxyl and superoxide radicals. In cells, superoxide radicals are rapidly transformed into H_2O_2 by action of superoxide dismutase. H_2O_2 has been shown to be implicated in HIF-1α protein induction (Chandel *et al.*, 2000; Gerald *et al.*, 2004; Brunelle *et al.*, 2005; Bell *et al.*, 2007; Pan *et al.*, 2007). We have previously demonstrated that utilization of catalase inhibited HIF-1α induction by Ang II in VSMCs (Richard *et al.*, 1999; Page *et al.*, 2002). To evaluate the possibility that after Ang II treatment H_2O_2 production in VSMCs is involved in decreasing PHD activity and HIF-1α hydroxylation, we studied the effect of H_2O_2 and catalase on the interaction of HIF-1α with pVHL. As seen in Figure 7A, the addition of exogenous H_2O_2 to VSMC extracts also inhibited pVHL binding to HIF-1α. Moreover, the pretreatment of VSMCs with catalase was able to restore pVHL binding to HIF-1α, which was decreased after Ang II treatment (Figure 7B). These results indicate that H_2O_2 is involved in decreasing HIF-1α hydroxylation and its subsequent binding to pVHL. We then hypothesized that the inhibition of HIF-1α hydroxylation and pVHL binding by H_2O_2 may be through the modulation of ascorbate levels in VSMCs observed after Ang II treatment. We therefore determined the effect of H_2O_2 on intracellular ascorbate concentrations. As shown in Figure 7C, VSMCs treated with H_2O_2 demonstrated decreased intracellular ascorbate levels. Additionally, catalase restored intracellular ascorbate levels decreased after Ang II treatment of VSMCs (Figure 7C). Taken together, our results indicate that in VSMCs, Ang II treatment–induced H_2O_2 production is responsible for decreases in intracellular ascorbate levels, which hampers PHD activity, leading to the stabilization of HIF-1α.

DISCUSSION

HIF-1 is a key regulator of gene induction by hypoxia in all mammalian cell types. Studies have clearly delineated that in VSMCs, different GPCR agonists, including Ang II and thrombin, are able to strongly increase the formation of the HIF-1 complex by inducing the HIF-1α subunit (Richard *et al.*, 2000; Gorlach *et al.*, 2001). Although the hypoxic activation of HIF-1 mainly implicates the stabilization of HIF-1α protein levels, GPCR agonists have devised an interesting and more complex mechanism to induce HIF-1α in normoxic conditions. VSMC stimulation with Ang II and thrombin: 1) increases the transcription of the HIF-1α gene, via the activation of diacylglycerol-sensitive PKC and nuclear factor (NF)-κB (Page *et al.*, 2002; Bonello *et al.*, 2007); 2) increases translational regulation of HIF-1α protein, via a ROS-dependent activation of the PI3K/p70S6K pathway (Page *et al.*, 2002); and 3) permits receptor tyrosine kinase transactivation that is necessary for increased HIF-1α protein translation and HIF-1 complex activation (Lauzier *et al.*, 2007). However, given the high instability of HIF-1α protein in normoxic conditions, it was unclear whether these effects alone were sufficient to increase HIF-1 levels. In this work, we demonstrate that Ang II also increases HIF-1α protein stabilization in VSMCs. Indeed, our studies clearly demonstrate that Ang II increases HIF-1α half-life. Ang II–treated cells increase HIF-1α protein stability by decreasing Pro^{402} hydroxylation, thereby attenuating pVHL binding to HIF-1α and diminishing HIF-1α ubiquitination and proteasome binding. Finally, we identify that Ang II–stimulated generation of H_2O_2 is responsible for decreasing intracellular ascorbate levels in VSMCs, an essential cofactor for the HIF-1α hydroxylation reaction. This work identifies novel and intricate signaling mechanisms that are intimately involved in HIF-1α protein regulation and activation after GPCR activation of VSMCs.

Recent evidence has emerged indicating that when induced in normoxic conditions, HIF-1α protein can also be stabilized. Hydroxylation by PHD enzymes is clearly defined as a major posttranslational modification implicated in HIF-1α stability (Bruick and McKnight, 2001; Epstein *et al.*, 2001; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Masson *et al.*,

2001). Certain normoxic inducers have been shown to regulate HIF-1α stability by modulating PHD enzyme activity. McMahon *et al.* (2006) have shown that PHD2 is down-regulated after a TGF-β1 treatment on different human cells, allowing the stabilization of HIF-1α. They demonstrated that TGF-β1 markedly and specifically decreases both mRNA and protein levels of PHD2 through the Smad signaling pathway. In VSMCs, Ang II stimulation has been shown to activate the Smad pathway (Rodriguez-Vita *et al.*, 2005; Wang *et al.*, 2006). However, we did not observe decreases in PHD2 mRNA or protein levels in VSMCs treated with Ang II. In opposition, PHD2 mRNA levels were even increased during the same time period (data not shown). This last result was expected because PHD2 is a HIF-1 target gene (Epstein *et al.*, 2001; Berra *et al.*, 2003; Metzen *et al.*, 2005). Knowles *et al.* (2006) have also demonstrated that normoxic stabilization of HIF-1α is mediated by a decrease in PHD enzyme activity. They show that PMA-induced macrophage differentiation also implicates HIF-1α induction via a down-regulation of intracellular labile iron pool, an essential co-factor of PHDs.

Our results clearly show that Ang II regulates HIF-1α hydroxylation. Studies by Chan *et al.* (2005) have shown that both of HIF-1α's hydroxylated proline residues are differentially regulated in hypoxic conditions. They demonstrated that Pro^{564} is the first residue to be hydroxylated when reaction conditions are favorable and that this modification promotes the subsequent hydroxylation of Pro^{402}. Together, the two hydroxylated proline residues potentiate the degradation of HIF-1α protein. It has been shown that modification of only one of these proline residues is sufficient for significant stabilization of HIF-1 (Chan *et al.*, 2005). Our results are in agreement with these observations and show that the treatment of VSMCs with Ang II decreases hydroxylation on Pro^{402}, resulting in decreased pVHL capture, while having no significant effect on Pro^{564} hydroxylation. Pro^{564} being the primary residue hydroxylated when all reaction conditions are filled, it is reasonable to believe that Pro^{402} will be the first residue to be regulated during conditions of cofactor depletion, which lead to decreased PHD activity.

Given the potency of ascorbate to inhibit increases in HIF-1α protein levels after Ang II treatment, we investigated the possibility that ascorbate could have inhibitory effects on mechanisms already described for HIF-1α induction by Ang II. First, the transcriptional modulations of HIF-1α by Ang II treatment were not inhibited by addition of ascorbate (see Supplementary Figure S2). Additionally, ROS production is an essential step for PI3K/p70S6K pathway activation by Ang II. PI3K/p70S6K pathway activation is involved in increasing HIF-1α translation during Ang II treatment (Page *et al.*, 2002). Although ascorbate is also known as an antioxidant compound, it had no inhibitory effect on the ROS-dependent activation of the PI3K/p70S6K pathway by Ang II. These observations converge on the potential role of ascorbate in the regulation of HIF-1α protein stability.

Oxygen and 2-oxoglutarate are cosubstrates, whereas ascorbate and Fe^{2+} are essential cofactors for PHD enzymes. Changes in the levels of these cofactors lead to the modulation of PHD enzymatic activity and its downstream effects on HIF-1α. Studies have shown that stimuli inducing HIF-1α, such as cobalt and nickel, modulate intracellular ascorbate levels to decrease hydroxylation and avoid degradation (Salnikow *et al.*, 2004; Karaczyn *et al.*, 2006). Vissers and Wilkie (2007) showed that HIF-1α is up-regulated in ascorbate-deficient neutrophils. Ascorbate has also been shown to enhance PHD enzyme activity and HIF degradation (Gorlach *et al.*, 2001; Knowles *et al.*, 2003; Vissers *et al.*, 2007). In the present study, we demonstrate that Ang II depletes VSMCs of ascorbate. During the hydroxylation reaction, Fe^{2+} bound to the enzyme is oxidized to Fe^{3+}, leading to the inactivation of catalytic activity. Ascorbate has been proposed to play the role of the reducing agent of Fe^{3+} to Fe^{2+} directly in the active site of the enzyme prolyl-4-hydroxylase, leading to the reactivation of the enzyme (de Jong *et al.*, 1982; Majamaa *et al.*, 1986). Decreased intracellular ascorbate may promote the oxidized Fe^{3+}-enzyme–bound state and cause a decrease in PHD enzyme activity.

It is now well documented that Ang II is a prooxidant hormone that increases ROS production in many cell types (Sachse and Wolf, 2007), including VSMCs (Kimura *et al.*, 2005; Zhang *et al.*, 2007; Griendling *et al.*, 1994; Lyle and Griendling, 2006). In these cells, the superoxide radical is transformed to H_2O_2 by action of superoxide dismutase (Clempus and Griendling, 2006). Our study shows that H_2O_2 contributes to the stabilization of HIF-1α protein through the regulation of intracellular ascorbate levels. Hydrogen peroxide is the major reactive moiety involved in Fenton reaction, which also implicates the oxidation of ferrous iron into ferric iron:

$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + Fe^{3+} + -OH$$

Additionally, it has been reported that enhanced ROS production promotes the inactivation of PHD and HIF stabilization, most probably via the Fenton reaction and oxidation of Fe^{2+} into Fe^{3+} (Gerald *et al.*, 2004; Bell *et al.*, 2007). Gerald *et al.* also demonstrate that ROS generation interferes with iron availability at the HIF prolyl-hydroxylase catalytic site, thereby inhibiting PHD activity in normal oxygen conditions. H_2O_2 has been implicated in the induction of HIF-1 and the transcriptional activation of target genes (Fandrey and Genius, 2000; Gorlach *et al.*, 2001; Mansfield *et al.*, 2005; Simon, 2006). Moreover, H_2O_2 has been shown to stabilize HIF-1α protein (Chandel *et al.*, 2000; Gerald *et al.*, 2004; Brunelle *et al.*, 2005; Bell *et al.*, 2007; Pan *et al.*, 2007). Because ascorbate is important in maintaining full activity of the enzyme by reducing ferric iron, ascorbate depletion in cells would contribute to HIF-1α stabilization in these conditions. Ascorbate itself may be consumed by oxidation in conditions where cells are exposed to peroxide or superoxide (Holmes *et al.*, 2000). In VSMCs, the precise mechanisms involved in H_2O_2-mediated decreases in ascorbate levels are presently unclear. However, our data, along with relevant published work, suggest that the treatment of VSMCs with Ang II leads to the generation of H_2O_2. The higher cellular level of H_2O_2 contributes to the oxidation of Fe^{2+} into Fe^{3+} via the Fenton reaction. Decreased Fe^{2+} availability leads to a decrease in PHD activity, decreased HIF-1α hydroxylation and increased HIF-1α stability. Increased Fe^{3+} causes augmented consumption of ascorbate for reduction to Fe^{2+}, further decreasing the ability of the cell to reduce Fe^{3+}. Supplementation of ascorbate in these conditions reverses the effect of H_2O_2 on iron availability, blocking the induction of HIF-1α by Ang II.

In conclusion, our study identifies novel signaling intermediates implicated in the regulation of the HIF-1 complex through nonhypoxic means. Ang II is a potent inducer of HIF-1α protein, and the present work identifies stabilization as a novel mechanism of HIF-1α protein induction by Ang II in VSMCs. This work contributes to the elucidation of Ang II–induced mechanisms for the activation of the HIF-1 complex and the subsequent activation of target genes. Given the importance and the evident implication of Ang II and the genes activated by the HIF-1 complex in different domains

of vascular biology, we believe that elucidation of pathways responsible for the regulation of the HIF-1 complex will have a very strong impact in vascular biology.

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