Molecular Pharmacology Fast Forward. Published on August 16, 2017 as DOI: 10.1124/mol.117.108654
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Selective HIF-1 Regulation under Nonhypoxic Conditions by the p42/p44 MAP Kinase Inhibitor, PD184161*

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Running title: HIF-1 regulation by PD184161

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Number of pages: 30
Number of figures: 7
Number of tables: 0
Number of references: 69

Word count:
Abstract: 242
Introduction: 570
Discussion: 1093

Abbreviations:
AngII, angiotensin II; $CoCl_2$, cobalt chloride; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; FBS, fetal bovine serum; GST, glutathione S-transferase; HA, hemagglutinin; HIF-1, hypoxia-inducible factor-1; HRE, hypoxic response element; MAPK, mitogen-activated protein kinase; mtROS, mitochondrial-derived reactive oxygen species; mito$K_{ATP,}$ mitochondrial ATP-sensitive potassium channel; ODDD, oxygen-dependent degradation domain; PHD, HIF prolyl-hydroxylase; PDGF, platelet derived growth factor; PVDF, polyvinylidene difluoride; RT, reverse transcription; S1P, sphingosine-1-phosphate; Thr, thrombin; VHL, von Hippel-Lindau tumor suppressor protein; VSMC, vascular smooth muscle cells.

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ABSTRACT

Hypoxia-inducible factor-1 (HIF-1) is a key gene regulator for cellular adaptation to low oxygen. In addition to hypoxia, several nonhypoxic stimuli, including hormones and growth factors, are an essential part for cell-specific HIF-1 regulation. Our studies have highlighted angiotensin II (AngII), a vasoactive hormone, as a potent HIF-1 activator in vascular smooth muscle cells (VSMC). AngII increases HIF-1 transcriptional activity by modulating specific signaling pathways. In VSMC, p42/p44 mitogen-activated protein kinase (MAPK) pathway activation is essential for HIF-1-mediated transcription during AngII treatment. The present study shows that PD184161, a potent MEK1/2 inhibitor, is a HIF-1 blocker in Ang II-treated VSMC. Unlike PD98059, a widely-used MEK1/2 inhibitor, we found that PD184161 blocked AngII-driven HIF-1α protein induction in a dose-dependent manner. Interestingly, the effect of PD184161 was specific to nonhypoxic activators, since HIF-1α induction by hypoxia (1% O_2) was unaffected under similar conditions. VSMC treatment with MG132, a proteasome inhibitor, indicated that PD184161 influenced HIF-1α protein stability. PD184161 also increased HIF-1α binding to VHL, a E3 ligase component and an indication of HIF-1α hydroxylation. Finally, we show that PD184161 blocked mitochondrial ROS (mtROS) production and cellular ATP levels, while enhancing ascorbate availability in AngII-treated VSMC. Taken together, our study indicates that, independently of p42/p44MAPK activation, PD184161 blocks mtROS generation by AngII, leading to reestablishment of cellular ascorbate levels, increased VHL binding and decreased HIF-1α stability. Therefore, this study reveals a previously unsuspected role for PD184161 as a HIF-1 inhibitor in VSMC under nonhypoxic conditions.

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Introduction

Hypoxia-inducible factor-1 (HIF-1) is an essential transcription factor for all cells that regulates cellular adaptation responses to low oxygen (O_2) availability (Semenza, 2003). Transcriptionally active HIF-1 is a heterodimeric complex composed of a stable HIF-1β subunit and an oxygen-sensitive HIF-1α subunit. HIF-1 is mainly regulated by proteasomal degradation, a mechanism under the control of oxygen-sensitive HIF prolyl hydroxylase domain-containing enzymes (PHD) (Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Kaelin and Ratcliffe, 2008). Under normal O_2, HIF-1α is hydroxylated on two conserved proline residues (P^{402} and P^{564} for human HIF-1) located within its oxygen–dependent degradation domain (ODDD). PHD activity and HIF-1α hydroxylation are dependent on obligate cofactors including O_2, 2-oxoglutarate, iron (Fe) and ascorbate (Schofield and Ratcliffe, 2005). Ascorbate rescues PHD activity following the hydroxylation reaction by reducing inactive Fe^{3+} to active Fe^{2+} (de Jong et al., 1982; Majamaa et al., 1986). HIF-1α hydroxylation promotes von Hippel-Lindau tumor suppressor protein (VHL)-directed HIF-1α polyubiquitination and subsequent proteasomal degradation (Cockman et al., 2000; Huang et al., 1998; Ivan et al., 2001; Jaakkola et al., 2001; Maxwell et al., 1999). When cofactor availability is modified, such as during low O_2, PHD activity and HIF-1α stability are directly altered. Stable HIF-1α can bind constitutive HIF-1β, forming the functional HIF-1 transcription complex. HIF-1 binds to specific hypoxia response elements (HRE), found within target genes promoters, resulting in adaptive gene activation. HIF-1 activation is also dependent on HIF-1α phosphorylation by p42/p44 mitogen-activated protein kinases (MAPK) (Fukuda et al., 2002; Hur et al., 2001; Lee et al., 2002; Minet et al., 2000; Richard et al., 1999; Sodhi et al., 2001; Sutton et al., 2007). HIF-1α phosphorylation permits Pin1 binding, a proline isomerase, which is indispensable for full HIF-1 transcriptional activity. (Han et al., 2016; Jalouli et al., 2014).

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Under normal O_2, HIF-1 is also activated by different cellular stimuli, a condition we described as nonhypoxic HIF-1 activation. Vascular smooth muscle cells (VSMC) show nonhypoxic HIF-1 activation following cell treatment with different receptor agonists, including angiotensin II (AngII), platelet derived growth factor (PDGF), sphingosine-1-phosphate (S1P), and thrombin (Thr) (Michaud et al., 2009; Richard et al., 2000). Distinct mechanisms come together for nonhypoxic HIF-1 activation in VSMC, including transcriptional and translational upregulation (Lauzier et al., 2007; Page et al., 2008; Page et al., 2002; Patten et al., 2010; Richard et al., 2000). HIF-1α protein stabilization is arguably the main mechanism for HIF-1 activation under these conditions. By stimulating mitochondrial-derived reactive oxygen species (mtROS) generation, AngII treatment causes a prooxidative intracellular microenvironment which leads to ascorbate depletion, PHD inhibition and finally HIF-1α stabilization (Page et al., 2008; Patten et al., 2010).

In this study, we show that PD184161 (5-bromo-2-[2-chloro-4-iodo-phenylamino]-N-cyclopropylmethoxy-3,4-difluoro-benzamide), abolishes AngII-induced HIF-1 activation in VSMC independently of p42/p44 MAPK activity. PD184161 is a potent and selective inhibitor of MEK1/2 (IC_{50} = 10-100 nM). Biologically active in plasma and more effective at inhibiting p42/p44 MAPK activation than other MEK1/2 inhibitors (PD098059, U0126), PD184161 has been useful for studying both the *in vitro* and *in vivo* roles of the Raf/MEK/MAPK pathway (Klein et al., 2006; Marshall et al., 2004; Thottassery et al., 2004). We demonstrate that PD184161 restores VHL-HIF-1α binding and HIF-1α degradation that was lost during AngII treatment. Finally, we show that PD184161 decreases AngII-induced mtROS generation and reestablishes cellular ascorbate levels, which is essential for HIF-1α hydroxylation and destabilization. Taken together, our work identifies PD184161 as a potent inhibitor of nonhypoxic HIF-1 induction in VSMC.

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Materials and Methods

Materials. AngII, cobalt chloride ($CoCl_2$), PD98059, PD184161, PDGF and thrombin were from Sigma-Aldrich (St-Louis, MO). S1P and MG132 were from EMD Millipore (Billerica, MA). MitoSOX and tetramethylrhodamine methyl ester (TMRM) were from ThermoFisher Scientific (Waltham, MA). SkQ1 was from Dr. Vladimir Skulachev at Moscow State University (Skulachev et al., 2009). Polyclonal anti-HIF-1α antibody was raised in our laboratory using rabbits immunized against the last 20 amino acids of the C-termini of the human protein (Richard et al., 1999). The monoclonal anti-phospho-p42/p44 MAPK antibody was from Sigma-Aldrich, whereas the polyclonal anti-p42/p44 MAPK was from EMD Millipore. Anti-glutathione S-transferase (GST) antibody was from Novus Biologicals (Littleton, CO). Monoclonal HA.11 antibody was from Covance (Emeryville, CA). Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were from Promega (Madison, WI). GST-HIF-1α fusion protein, VHL-hemagglutinin (HA), luc-HIF-1α-ODDD constructs and pHIF-1A-572/+32Luc reporter vector were kind gifts from Drs. Jacques Pouysségur (University of Nice-Sophia Antipolis), Peter Ratcliffe (University of Oxford), Richard K. Bruick (University of Texas Southwestern) and Gregg Semenza (Johns Hopkins University, Baltimore, MD), respectively.

Cell culture. VSMC were isolated from thoracic aortas of 6-week-old male Wistar rats by enzymatic dissociation (Owens et al., 1986). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 12.5% fetal bovine serum (FBS) and supplemented with antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin) and 2 mM glutamine (ThermoFisher Scientific). In all experiments, cells were deprived of serum 16 h prior to treatment. Hypoxic conditions were achieved by incubating cells in a sealed hypoxic workstation (Baker Ruskinn, Bridgend, UK). Oxygen levels in the workstation were maintained at 1% with a residual gas mixture containing 94% nitrogen and 5% CO_2.

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Western blot analysis. VSMC were lysed in 2× Laemmli sample buffer. Lowry assay was used to determine protein concentration. Cell lysates were resolved on SDS-polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Immobilon-P; EMD Millipore). Proteins of interest were analyzed using the indicated antibodies and visualized with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) or with enhanced chemiluminescence (ECL) system (GE Healthcare Life Sciences, Piscataway, NJ). Western blots were quantified using Odyssey Application software v.3.1 (LI-COR). Results are representative of at least three independent experiments.

Real-time quantitative RT-PCR (qRT-PCR). RNA was isolated from VSMC using TRizol per the manufacturer's protocol (ThermoFisher Scientific). RNA purity was assessed by Nanodrop (ThermoFisher Scientific). Reverse transcription (RT) was performed with qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). qRT-PCR was performed on a MX3005P system (Agilent Technologies, Santa Clara, CA) using Perfecta SYBR Green SuperMix, Low ROX kit (Quanta Biosciences). The primer pair used for rat HIF-1α (accession no. NM_024359) was fwd: 5'-CTTCTGATGGAAGCACTAGACAA-3', rev: 5'-TTCCAAGTCTAAATCAGTGTCCT-3'. Rat HPRT1 (accession no. NM_012583) was used as a reference gene (fwd: 5'-CAGTCCCAGCGTCGTGATTAGT-3', rev: 5'-ATCCAGCAGGTCAGCAAAGAAC-3'). The expression of each gene of interest relative to HPRT1, a reference gene, was calculated based on the threshold cycle (Ct) using the Pfaffl formula (Pfaffl, 2001). Results are presented as a percent change calculated by comparing the treated sample with its corresponding control condition as indicated.

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Luciferase assay. Cells were seeded in 12-well plates and transfected by Superfect transfection reagent (Qiagen, Valencia, CA) at a 1:3 DNA/reagent ratio with 2 μg/well of either luc-HIF-1α-ODDD or pHIF-1A-572/+32Luc luciferase reporter vectors. *Renilla reniformis* luciferase expression vector (25 ng/well) was used to control transfection efficiency. 48 h post-transfection, cells were serum-deprived for 16 h and treated as indicated. The Dual Luciferase Reporter Assay System (Promega) was used for luciferase assays. Measurements were performed on a Luminoskan Ascent microplate reader with integrated injectors (ThermoFisher Scientific). Results are expressed as a ratio of firefly luciferase activity over *Renilla reniformis* luciferase activity. Experiments are an average ± SD of triplicate data representative of three independent experiments.

VHL capture assay. VHL capture assay was performed as previously described (Page et al., 2008). Briefly, cells were grown to confluence, deprived of serum for 16 h, treated as indicated and then lysed. Cytoplasmic extracts (250 μg) were incubated with Sepharose-bound GST-HIF-1α (50 μg) for 1 h at room temperature and then washed with NETN buffer prior to incubation with *in vitro*-translated VHL-HA in NETN buffer overnight at 4ºC with rotation. Sepharose-bound GST-HIF-1α was then washed with NETN buffer and suspended in 2× Laemmli sample buffer. Samples were resolved in SDS-polyacrylamide gels, transferred to PVDF and revealed using Western blot analysis with specific antibodies as indicated.

mtROS assay. Mitochondrial ROS production was determined using MitoSOX Red mitochondrial superoxide indicator (ThermoFisher Scientific) which is selectively targeted to the mitochondria and is fluorescent upon ROS oxidation. MitoSOX was used per manufacturer's protocol. Briefly, cells seeded on glass-bottomed cell culture dishes, were serum-deprived overnight in phenol red-free DMEM and

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then incubated with MitoSOX (1 μM) for 1 h before imaging. VSMC were treated with or without PD184161 for 20 min prior to incubation with AngII during the final 20 min. Cell imaging was performed with FV1000 confocal microscope equipped with a live cell apparatus (60× oil, 1.4 NA) driven by FluoView software (Olympus, Tokyo, Japan). Fluorescence quantification was performed using the Measure Integrated Density function of ImageJ (imagej.nih.gov).

Intracellular ascorbate assay. Ascorbate levels were analyzed by spectrophotometry using a modified protocol (Queval and Noctor, 2007). Briefly, VSMC were cultured in DMEM supplemented with 250 μM ascorbate. Upon reaching confluence, cells were serum-deprived for 16 h in ascorbate-supplemented DMEM. Fresh DMEM without ascorbate was added 1 h prior to treatment. Cells were washed twice with PBS, lysed in methanol, briefly sonicated and centrifuged at 20,000×g. Samples were diluted in 0.2 mM NaH_2PO_4 pH 5.6 and absorbance was measured at 265 nm. Ascorbate peroxidase (0.4 U) was then added to samples for 15 min, and the absorbance was again measured at 265 nm. Ascorbate concentrations were determined as the difference in absorbance before and after addition of ascorbate peroxidase. A Lowry protein assay was used for sample normalization.

ATP assay. VSMC were serum-deprived overnight in low glucose DMEM. Cells were treated as indicated and intracellular ATP levels were measured with a luminescent ATP detection kit (Abcam, Cambridge, UK) according to the manufacturer's protocol using a Luminoskan Ascent microplate reader. A Lowry protein assay was used for sample normalization.

Mitochondrial membrane potential ($\Delta\Psi_M$) measurements. $\Delta\Psi_M$ was determined using tetramethylrhodamine methyl ester. VSMC pretreated as indicated prior to incubation with TMRM (200

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nM) for 20 min. Cells were then washed and placed in phenol red-free DMEM. Live cell imaging was performed with an AxioObserver Z1 system using a 40× objective and a Axiocam MRm camera controlled by Zen 2 software (Carl Zeiss Microscopy, Oberkochen, Germany). Fluorescence quantification was performed using the Measure Integrated Density function of ImageJ (imagej.nih.gov).

Statistical analysis. GraphPad Prism 5 software was used for statistical analyses of experiments. Statistical analysis on Fig. 1C and Fig. 4A were performed using a one-way repeated measures ANOVA with a Newman–Keuls *post hoc* test. Statistical analysis on Figs. 1A, 2B, 2D, 4B, 5B, 6 and 7B and 7C were performed using a two-way repeated measures ANOVA with a Bonferroni correction. Statistical analysis on Fig. 3B was performed using a three-way ANOVA with the Benjamini–Hochberg procedure. Results were deemed significant if they attained a 95% confidence level ($p < 0.05$) and all comparisons were performed with an experiment-wise error rate (EER) of 0.05.

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Results

PD184161 inhibits HIF-1α protein induction induced by AngII in VSMC. p42/p44 MAPK activation is essential for HIF-1 function (Mylonis et al., 2006; Richard et al., 1999; Sutton et al., 2007). In VSMC, HIF-1 is strongly induced by AngII, independently of oxygen availability (Richard et al., 2000). Through epidermal growth factor (EGF) receptor transactivation, Ang II is a potent activator of p42/p44 MAPK in VSMC and HIF-1 activity is strongly linked to p42/p44 MAPK activation (Lauzier et al., 2007). However, HIF-1α protein induction is only modestly affected by p42/p44 MAPK pathway inhibitors, such as PD98059. To more clearly investigate the role of p42/p44 MAPK in AngII-induced HIF-1 activation in VMSC, we assayed a potent inhibitor of the p42/p44 MAPK pathway, PD184161. This MEK1/2 inhibitor was shown to strongly inhibit p42/p44 MAPK signaling at concentrations lower than 1 μM (Klein et al., 2006). Theoretically, PD184161 should have fewer off target effects. However, we were surprised to discover that VSMC pretreatment with low concentrations of PD184161 blocked HIF-1α accumulation by AngII in a dose-dependent manner (Fig. 1A). As expected, VSMC pretreatment with PD98059 had only minor inhibitory effects on HIF-1α induction by AngII (Fig. 1B and 1C). It is important to note that the concentrations used for both inhibitors demonstrate similar effects on p42/p44 MAPK phosphorylation levels (Fig. 1B). These results indicate that PD184161 possesses a potent inhibitory effect on HIF-1α protein accumulation when VSMC are treated with AngII. The effect of PD184161 on HIF-1α is independent of p42/p44 MAPK pathway activation.

PD184161 blocks nonhypoxic HIF-1α induction. Our previous studies have shown that in VSMC, AngII activates HIF-1 through mechanisms that differ from hypoxia (1% O_2) (Lauzier et al., 2007; Page et al., 2008; Page et al., 2002; Patten et al., 2010). We wanted to determine whether the effect of PD184161 on HIF-1α induction was specific to cells treated in nonhypoxic conditions. VSMC were

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pretreated with PD184161 and then incubated under hypoxic (1% O_2) or treated with $CoCl_2$, a potent inhibitor of HIF-1 hydroxylation which mimics hypoxia. As seen in Fig. 2A and 2B, while PD184161 pretreatment blocked HIF-1α protein induction by AngII, it was ineffective in blocking HIF-1α protein induction by 1% O_2 or $CoCl_2$ treatment. Other nonhypoxic activators are also known to induce HIF-1α in VSMC. To determine whether HIF-1α induction by other nonhypoxic stimuli is also affected by PD184161, we treated cells with thrombin and sphingosine-1-phosphate. Thr and S1P both induce HIF-1α protein levels through similar mechanisms to those observed with AngII (Michaud et al., 2009; Richard et al., 2000). As seen in Fig. 2C and 2D, PD184161 also decreased HIF-1α accumulation by both Thr and S1P treatment in a similar fashion to that observed with AngII-treated VSMC. Similar results were also observed when VSMC where treated with PDGF (Supplemental Figure 1). Taken together, our results indicate the selective inhibition of nonhypoxic HIF-1α protein induction by PD184161 in VSMC.

PD184161 blocks AngII-induced HIF-1α stabilization. Since AngII modulates HIF-1 activation through different mechanisms, we first wanted to determine whether PD184161 treatment alters HIF-1α gene transcription. To undertake these experiments, we evaluated the effect of PD184161 on HIF-1α mRNA expression by performing qRT-PCR experiments. As seen in Supplemental Figure 2, our results show that PD184161 had no significant effect on HIF-1α mRNA levels either in the presence or absence of AngII. Additionally, PD184161 did not significantly affect expression levels of a reporter driven by the minimal HIF-1α gene promoter (pHIF-1A-572/+32Luc, results not shown) in the presence or absence of AngII. Taken together, these results indicate that PD184161 does not modify HIF-1α levels by blocking HIF-1α gene transcription.

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As mentioned previously, regulating HIF-1α stability is the main mechanism by which AngII activates HIF-1 in VSMC (Page et al., 2008; Patten et al., 2010). We therefore investigated the impact of PD184161 on HIF-1α protein stability by using MG132, a widely-used proteasome inhibitor. As expected, VSMC pretreatment with MG132 blocked HIF-1α degradation and led to its accumulation in VSMC treated with or without AngII (Fig. 3A and 3B). Interestingly, while HIF-1α protein levels were significantly reduced by PD184161 during AngII treatment, it was ineffective in the presence of MG132. This result indicated that in VSMC, PD184161 affected AngII-mediated changes in HIF-1α protein stability prior to proteasomal degradation. VHL binding to hydroxylated HIF-1α is a decisive step for HIF-1α proteasomal degradation (Cockman et al., 2000; Huang et al., 1998; Ivan et al., 2001; Jaakkola et al., 2001; Maxwell et al., 1999). Since we have previously shown that in VSMC, AngII treatment increases HIF-1α stability by blocking PHD activity and subsequent VHL binding, we decided to investigate the effect of PD184161 on VHL-dependent HIF-1α degradation (Page et al., 2008). First, we performed a VHL capture assay to determine HIF-1α hydroxylation levels. A GST-HIF-1α fusion protein, comprising amino acids 344-582 from human HIF-1α, was incubated with extracts of cells treated with AngII in the presence or absence of PD184161. These extracts were then incubated with *in vitro*-translated HA-tagged VHL. As seen in Fig. 4A and 4B, VHL binding to HIF-1α was significantly reduced in AngII-treated VSMC as compared to untreated cells. As expected, the potent hydroxylase inhibitor $CoCl_2$ completely blocked VHL binding to hydroxylated HIF-1α. More interestingly, decreased VHL binding to HIF-1α observed under AngII treatment was reversed in cells pretreated with increasing concentrations of PD184161. Second, we used the luc-HIF-1α ODDD construct in a luciferase assay to further determine how PD184161 regulates HIF-1α stability. Luc-HIF-1α ODDD encodes a fusion protein between firefly luciferase and HIF-1α's oxygen dependent degradation domain (ODDD). This construct contains two proline residues targeted for oxygen-dependent hydroxylation and, consequently,

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VHL binding (Salnikow et al., 2004). VSMC were transfected with luc-HIF-1α ODDD, pretreated with PD184161 and then treated with AngII. As expected, AngII significantly increased luciferase activity in VSMC as compared to untreated cells (Fig. 4C). More importantly, PD184161 pretreatment significantly blocked AngII-dependent luciferase activity in a dose-dependent manner. Of note, PD184161 had no effect on $CoCl_2$-dependent luciferase activity using this same construct (results not shown). Taken together, these results indicate that PD184161 inhibits HIF-1α stabilization during Ang II treatment by reestablishing HIF-1α hydroxylation and VHL binding to the ODDD.

PD184161 inhibits AngII-induced mitochondrial ROS generation and ascorbate levels in VMSC. Our previous studies showed that increased mtROS production is responsible for decreasing cellular ascorbate levels and inhibiting PHD activity during AngII treatment (Page et al., 2008; Patten et al., 2010). Therefore, we wanted to determine whether PD184161 could also block AngII-induced mtROS production. Using MitoSOX, a mitochondrial-targeted probe for detecting ROS, VSMC treated with AngII showed increased mtROS levels as compared to untreated cells (Fig. 5A and 5B). More importantly, VMSC pretreated with PD184161 showed a striking inhibition of mtROS levels. This result suggests that PD184161 decreases AngII-induced HIF-1α stabilization by reducing mtROS generation in VSMC. Our previous work showed that cellular ascorbate levels are modified and closely linked to mtROS generation and HIF-1 activation in VSMC (Page et al., 2008; Patten et al., 2010). We therefore wanted to determine whether PD184161 could block decreased cellular ascorbate levels observed during AngII treatment. Using a protocol based on differential spectrometric measurements, AngII treatment significantly decreased cellular ascorbate levels in VSMC (Fig. 6). When VSMC were treated with SkQ1, a mitochondrial-targeted antioxidant, decreased intracellular ascorbate levels observed during AngII treatment were reestablished. More importantly, pretreatment of VSMC with increasing concentrations of PD184161 restored intracellular ascorbate levels under AngII treatment. Taken

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together, our results indicate that PD184161 abrogates mtROS production by AngII treatment in VSMCs and reestablishes intracellular ascorbate levels and HIF-1α destabilization under nonhypoxic conditions.

PD184161 inhibits mitochondrial membrane potential depolarization and ATP production in AngII-treated VMSC. Depolarization of mitochondrial membrane potential ($\Delta\Psi_M$), is known to influence mtROS generation in VSMC (Kimura et al., 2005a). To determine if PD184161 affects $\Delta\Psi_M$ in AngII-treated VSMC, we analyzed $\Delta\Psi_M$ using TMRM. As seen in Fig. 7A and 7B, VSMC treated with AngII showed decreased TMRM staining as compared to untreated cells, an indication $\Delta\Psi_M$ depolarization. More interestingly, pretreatment of VSMC with PD184161 restored $\Delta\Psi_M$ under AngII treatment. These results indicate that the maintenance of mitochondrial membrane potential by PD184161 may be responsible for inhibition of mtROS generation.

ATP is essential for the opening of mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}), a main mediator of $\Delta\Psi_M$ depolarization. PD184161 was previously shown to affect ATP production (Yung et al., 2004). As seen in Fig. 7C, VSMC pretreated with PD184161 show decreased ATP production in comparison to untreated cells. These results indicate that PD184161 may decrease HIF-1 levels induced under nonhypoxic conditions by modifying both ATP production and $\Delta\Psi_M$ depolarization. These two phenomena could therefore reestablish mtROS generation and cellular ascorbate levels to those seen in untreated cells.

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Discussion

By blocking the p42/p44 MAPK signaling pathway, MEK 1/2 inhibitors, such as PD98059, act as negative regulators of HIF-1 transcriptional activity (Fukuda et al., 2002; Hur et al., 2001; Lee et al., 2002; Minet et al., 2000; Richard et al., 1999; Sodhi et al., 2001; Sutton et al., 2007). The present study demonstrates that PD184161, another potent MEK 1/2 inhibitor, is also a selective inhibitor of nonhypoxic HIF-1α protein accumulation in VSMC. More specifically, we show that PD184161 strikingly decreased HIF-1α protein levels during AngII treatment by blocking associated mtROS generation, reestablishing cellular ascorbate levels, restoring proteasomal targeting by VHL, and finally, HIF-1α proteasomal degradation.

In VSMC, AngII is a strong activator of the p42/p44 MAPK pathway through EGFR transactivation as well as being a potent HIF-1 inducer (Lauzier et al., 2007). p42/p44 MAPK pathway activation by AngII enhances HIF-1 transcriptional activity by promoting direct HIF-1α phosphorylation (Lauzier et al., 2007). Pin1, a proline isomerase, can recognize this phosphorylation, modify HIF-1α conformation and enhance HIF-1-related gene transcription (Jalouli et al., 2014). However, in AngII-treated VSMC and other systems, p42/p44 MAPK pathway activation has only modest effects on HIF-1α protein induction (Berra et al., 2001; Lauzier et al., 2007). In fact, inhibition of EGFR and p42/p44 MAPK activation by AG1478 or PD98059, respectively, only cause a slight decrease in AngII-increased HIF-1α levels. Consistent with these studies, we show here that unlike PD184161, PD98059 only modestly affects HIF-1α induction by AngII in conditions where p42/p44 MAPK activation is completely abolished. Given the potency of PD184161 to block HIF-1α induction during VSMC stimulation with AngII, our results indicate that PD184161 influences HIF-1α protein levels through a mechanism distinct from its inhibitory effect on p42/p44 MAPK.

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In addition to AngII, other nonhypoxic stimuli, such as Thr, S1P and PDGF, are also able to induce HIF-1α protein levels in VSMC through similar mechanisms. Our present study clearly shows that PD184161 selectively abolishes nonhypoxic HIF-1α induction without interfering with hypoxic HIF-1 induction. We decided to investigate how this compound negatively impacts mechanisms involved in nonhypoxic HIF-1 induction. Our previous studies demonstrated that the main mechanism by which AngII induces HIF-1α protein is through the inhibition of PHD enzymatic activity, which leads to decreased HIF-1α hydroxylation, VHL binding, ubiquitination, and proteasomal degradation (Page et al., 2008). mtROS generation is important for AngII-induced HIF-1α, most probably by the Fenton reaction (Fe2+ oxidation to Fe3+) resulting in decreased intracellular ascorbate levels (Bell et al., 2007; Gerald et al., 2004). Indeed, inhibition of mtROS production was shown to negatively regulate AngII-induced HIF-1α accumulation (Page et al., 2008; Patten et al., 2010). Accordingly, we show here that PD184161 promotes HIF-1α proteasomal degradation, increased hydroxylation and VHL binding, inhibition of AngII-induced mtROS generation production, and the reestablishment of intracellular ascorbate availability. Previous studies demonstrated that p42/p44 MAPK activity is not implicated in the HIF-1α stability (Berra et al., 2001). This supports our findings that the effect of PD184161 on HIF-1α protein induction is independent of p42/p44 MAPK pathway inhibition.

The mitochondrial respiratory chain represents a major source of ROS (Camara et al., 2010; Dedkova et al., 2013; Drose, 2013; Page et al., 2008; Wojtovich et al., 2013). Our previous work demonstrated that HIF-1α induction by AngII requires mitochondrial/complex III generated mtROS (Patten et al., 2010). Hence, PD184161 may reduce AngII-mediated HIF-1α accumulation by blocking mitochondrial respiratory chain function. Interestingly, unlike PD98059, PD184161 can induce a profound ATP depletion by modulating ATP synthase activity, in a similar manner to oligomycin A, a mitochondrial ATP synthase inhibitor (Yung et al., 2004). ATP synthase inhibition, and thus changes in oxidative

Molecular Pharmacology Fast Forward. Published on August 16, 2017 as DOI: 10.1124/mol.117.108654
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phosphorylation, was shown to negatively regulate ROS production (Santamaria et al., 2006). A second study indicated that in addition to inhibiting MEK 1/2, PD184161 caused cell death in glucose-deprived cells, a result of profound ATP depletion caused by ATP-synthase inhibition (Yung et al., 2004). Accordingly, we show decreased ATP levels under PD184161 treatment in VSMC. It is therefore likely that reduced AngII-induced mtROS generation under PD184161 treatment is a result of decreased ATP synthase activity and lower ATP levels. In addition, depolarization of mitochondrial membrane potential through the opening of mitoKATP was shown to be implicated in AngII-induced mtROS generation (Kimura et al., 2005a). It was reported that kinase inhibitors can affect protein function and act as competitors for ATP binding proteins by forming aggregates (Bain et al., 2003; Davies et al., 2000; Genini et al., 2000; Halestrap, 1999; Hanks et al., 1988; McGovern and Shoichet, 2003; Xue et al., 2002). We show that PD184161 exerts a negative effect on mitochondrial membrane depolarization in response to AngII treatment in VSMC. This further indicates that decreased ATP, mitoKATP channel activity, and depolarization of the $\Delta\Psi M$ are potentially involved in decreased mtROS generation and decreased HIF-1 induction in VSMCs during nonhypoxic activation.

AngII is an important regulator for many physiopathological processes such as hypertension, atherosclerosis, VSMC remodeling, and fibrosis (Hanna et al., 2002; Heeneman et al., 2007; Touyz, 2003). p42/p44 MAPK pathway activation is implicated in mediating the effects of AngII (Ishida et al., 1998; Ishida et al., 1999; Queval and Noctor, 2007; Wang et al., 2015). By activating genes involved in the control of several cell functions, HIF-1 is also implicated in number of AngII-induced degenerative diseases, such as cardiovascular and kidney diseases (Imanishi et al., 2014; Luo et al., 2015; Obama et al., 2015; Zhu et al., 2011). Because of its important role in disease progression, HIF-1 targeting has gained significant interest. Our study demonstrates PD184161 is a potent inhibitor of AngII-induced HIF-1α protein induction and identifies this compound as a potential treatment strategy. PD184161 has

an added advantage since it inhibits HIF-1α by blocking mtROS production, an important mediator of AngII-activated responses (Caldiz et al., 2011; Caldiz et al., 2007; De Giusti et al., 2008; De Giusti et al., 2009; Doughan et al., 2008; Kimura et al., 2005a; Kimura et al., 2005b; Patten et al., 2010). In addition, mice expressing mitochondria-targeted catalase were resistant to AngII-dependent cardiac hypertrophy, autophagy, fibrosis and mitochondrial damage (Dai and Rabinovitch, 2011). Therefore, we believe that PD184161 may be therapeutically interesting for further studies concerning the physiopathological roles of AngII.

In summary, our study identifies PD184161 as a robust inhibitor of nonhypoxic HIF-1 induction. PD184161 is a potent p42/p44 MAPK pathway inhibitor, and the present work now identifies it as a HIF-1α inhibitor, by way of blocking mtROS generation in VSMC. Given the importance implication of p42/p44 MAPK pathway, ROS, and HIF-1 in several AngII-mediated physiological and physiopathological processes, we believe that these studies have an interesting impact in AngII-related vascular biology.

Molecular Pharmacology Fast Forward. Published on August 16, 2017 as DOI: 10.1124/mol.117.108654
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Acknowledgements

We thank Drs Jacques Pouysségur, Peter Ratcliffe, Richard K. Bruick and Gregg Semenza for the GST-HIF-1α, VHL-HA, luc-HIF-1α ODDD constructs and pHIF-1A-572/+32Luc reporter vector, respectively. We would also like to thank Dr Josée N. Lavoie for technical support in using the Zeiss microscope.

Authorship Contributions

Participated in research design: Jalouli, Richard.

Conducted experiments: Jalouli, Mokas, Turgeon, Lamalice.

Performed data analysis: Jalouli, Mokas, Lamalice, Richard.

Wrote or contributed to the writing: Jalouli, Mokas, Turgeon, Lamalice, Richard.

Disclosure

All authors declare no competing interests.

Molecular Pharmacology Fast Forward. Published on August 16, 2017 as DOI: 10.1124/mol.117.108654
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Molecular Pharmacology Fast Forward. Published on August 16, 2017 as DOI: 10.1124/mol.117.108654
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Footnotes

*This work was supported by research grants to D.E.R. from the Canadian Institutes of Health Research (CIHR; MOP-102760) and the Heart and Stroke Foundation of Québec. S.M. and C.A.T. received graduate scholarships from the Fonds de recherche du Québec - Santé (FRQS).

Molecular Pharmacology Fast Forward. Published on August 16, 2017 as DOI: 10.1124/mol.117.108654
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Figure legends

Figure 1. AngII-induced HIF-1α is inhibited by PD184161, an inhibitor of p42/p44 MAPK activation. (A) Quiescent VSMC were pretreated or not with PD184161 (at indicated concentrations) for 20 min and then treated with AngII (100 nM) for 4 h. Total extracts were resolved by SDS-PAGE and Western blotted with HIF-1α, phospho-p42/p44 (POp42/p44), and total-p42/p44 (p42/p44) antibodies. Western blot experiments were quantified (upper panel) using the Odyssey Infrared Imaging System. Results are expressed as a ratio of the amount of HIF-1α protein to the amount of total-p42/p44 protein and are an average with SD of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to indicated conditions. (B) Quiescent VSMC were pretreated or not with PD184161/PD98059 (at indicated concentrations) for 20 min and then treated with AngII as previously described. C) Western blot experiments were quantified using the Odyssey Infrared Imaging System. Results are expressed as a ratio of the amount of HIF-1α protein to the amount of total-p42/p44 protein and are an average with SD of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to cells treated with AngII only.

Figure 2. HIF-1α induction is selectively regulated by PD184161. (A) Quiescent VSMC were pretreated or not with PD184161 (at indicated concentrations) and then were treated with AngII (100 nM), 1% O_2 or cobalt ($CoCl_2$, 200 μM) for 4 h. Samples were resolved by SDS-PAGE and Western blotted with HIF-1α, phospho-p42/p44 (POp42/p44) and total-p42/p44 (p42/p44) antibodies. (B) Western blot experiments were quantified using the Odyssey Infrared Imaging System. Results are expressed as a ratio of the amount of HIF-1α protein to the amount of total-p42/p44 protein and are an average with SD of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to treated cells without PD184161. (C) Quiescent VSMC were pretreated or not with PD184161 (50 nM) for 20 min and then

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treated with 1% O_2, AngII (100 nM), Thr (5 U/ml) and S1P (2 μM) for 4 h. Western blot was then used to analyze HIF-1α, POp42/p44 and p42/p44 levels. D) Western blot experiments were quantified using the Odyssey Infrared Imaging System. Results are expressed as a ratio of the amount of HIF-1α protein to the amount of total-p42/p44 protein and are an average with SD of three independent experiments. *$p < 0.05$ and **$p < 0.01$ as compared to indicated conditions.

Figure 3. Effect of PD184161 on HIF-1α proteasomal degradation. (A) Quiescent VSMC were pretreated or not with MG132 (10 μM) for 20 min prior to the addition or not of PD184161 (at indicated concentrations) for 20 min. VSMC were then treated with AngII (100 nM) for 4 h. Samples were resolved by SDS-PAGE and Western blotted with antibodies against HIF-1α and total-p42/p44 (p42/p44). (B) Western blot experiments were quantified with the Odyssey Infrared Imaging System using total-p42/p44 as a loading control. Results are expressed as the percentage of HIF-1α levels compared to either vehicle + AngII or to MG132 + AngII treated cells and are an average with SD of three independent experiments. *$p < 0.05$ and ***$p < 0.001$ as compared to indicated conditions.

Figure 4. PD184161 enhances HIF-1α hydroxylation and VHL binding. (A) Quiescent VSMC were pretreated or not with PD184161 (at indicated concentrations) for 20 min. VSMC were then treated with AngII (100 nM) or $CoCl_2$ (200 μM) for 4 h. GST-HIF-1α coupled to sepharose beads were incubated with cytoplasmic extracts for 1 h followed by *in vitro*-translated VHL for 16 h. Precipitates were resolved by SDS-PAGE and Western blotted with HA (VHL) and GST antibodies. Western blot experiments were quantified (B) with the Odyssey Infrared Imaging System software using GST-HIF-1α as a loading control. Results are expressed as the percentage of ratio of VHL levels compared to GST-HIF-1α levels and are an average with SD of three independent experiments. **$p < 0.01$ and ***$p < 0.001$ as compared

to untreated cells (vehicle only). (C) VSMC were transfected with 2 μg of CMV-luc-HIF-1α-ODDD and 25 ng of *Renilla reniformis* luciferase expression vectors. 48 h post-transfection, quiescent VSMC were pretreated or not with PD184161 (at indicated concentrations) for 20 min and treated with AngII (100 nM) for 4 h. Cells were then lysed and luciferase activity was measured. Results are expressed as a ratio of firefly luciferase activity to *R. reniformis* luciferase activity and are an average with SD of three independent experiments performed in triplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to indicated conditions.

Figure 5. PD184161 influences mtROS production by AngII. (A) VSMC, seeded on 35-mm glass-bottomed cell culture dishes, were incubated with MitoSOX (1 μM) for a total of 1 h. 20 min after adding MitoSOX, cells were pretreated or not with PD184161 (50 nM). 20 min before imaging, VSMC were treated with AngII (100 nM). Differential interference contrast (DIC) was used for whole cell imaging and subsequent quantification. B) Quantification of measurements in A are expressed as arbitrary units of MitoSOX fluorescence and are an average with SD of three independent experiments, n = 50 cells. $^{***}p < 0.001$ as compared to untreated VSMC. †† $p < 0.01$ as compared to indicated conditions.

Figure 6. PD184161 effects intracellular levels of ascorbate. Quiescent VSMC were cultured in ascorbate-supplemented DMEM and pretreated or not with PD184161 (at indicated concentrations) or SkQ1 (250 nM). VSMC were then treated with AngII (100 nM) for 4 h. Cells were washed twice with PBS and resuspended in methanol. Ascorbate concentrations were determined using a protocol based on differential spectrometric measurements (see Materials and methods). Results are expressed as the percentage of relative intracellular ascorbate levels compared to untreated cells and are an average with SD of three independent experiments. $^{***}p < 0.001$ as compared to untreated cells.

Molecular Pharmacology Fast Forward. Published on August 16, 2017 as DOI: 10.1124/mol.117.108654
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Figure 7. PD184161 inhibits mitochondrial membrane potential depolarization and ATP production. (A) VSMC, seeded on 35-mm glass-bottomed cell culture dishes, were cells were pretreated or not with PD184161 (50 nM) for 20 min and treated with AngII (100 nM) for 40 min. Cells were then incubated with TMRM (200 nM) for 20 min before imaging. B) Quantification of measurements in A are expressed as arbitrary units of TMRM fluorescence and are an average with SD of three independent experiments, n = 50 cells. $^{**}p < 0.01$ as compared to untreated VSMC. (C) Quiescent VSMC were pretreated or not with PD184161 (50 nM) for 20 min and then treated with AngII (100 nM) for 1 h. ATP levels were measured as described in Materials and Methods. Results are expressed as the percentage of relative ATP levels compared to untreated cells and are an average with SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to untreated cells.

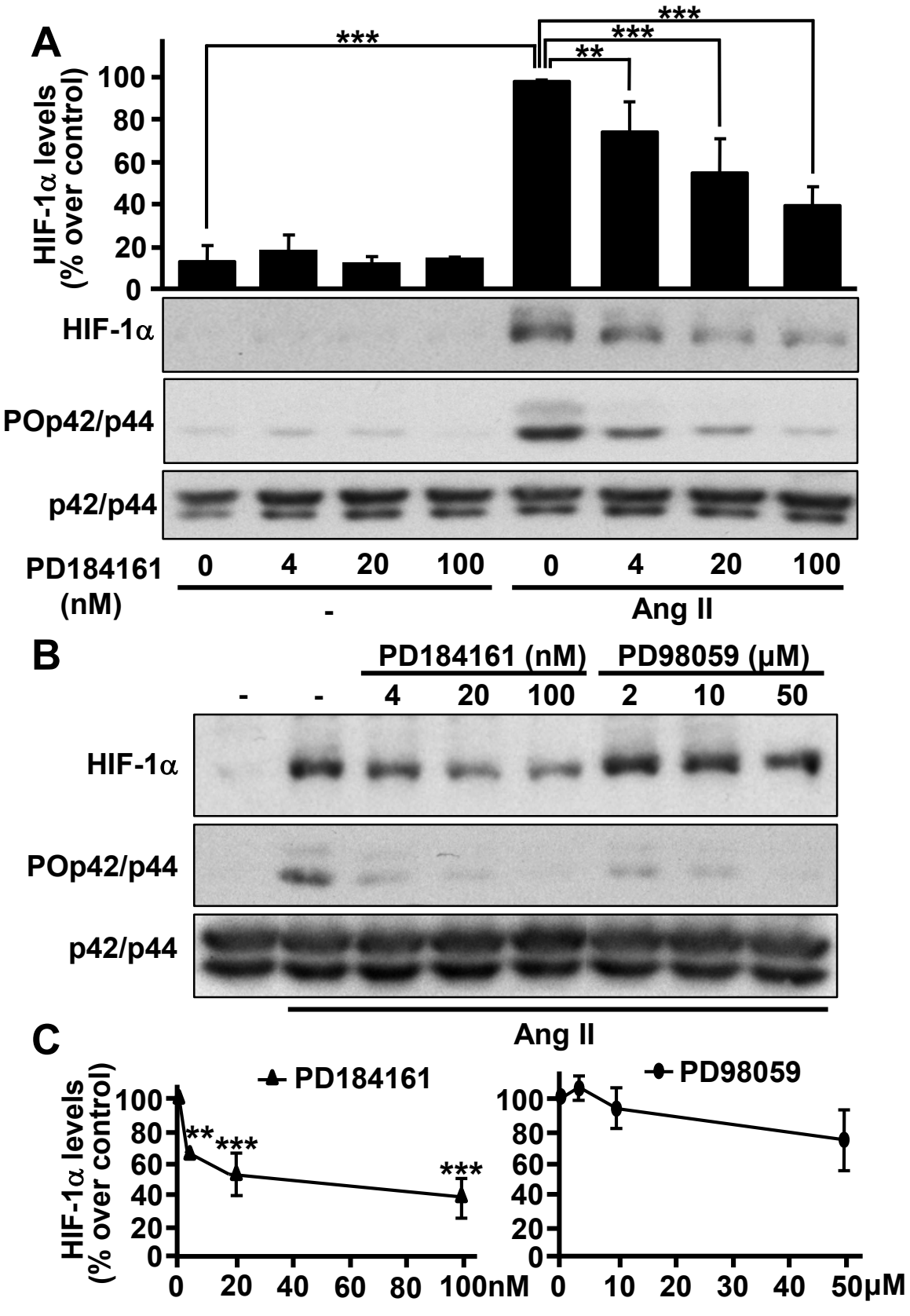
A
HIF-1α levels (% over control)
***
***
***
**
HIF-1α
POp42/p44
p42/p44
PD184161 (nM)
0 4 20 100 0 4 20 100
-
Ang II
B
PD184161 (nM)
PD98059 (μM)
- - 4 20 100 2 10 50
HIF-1α
POp42/p44
p42/p44
Ang II
C
HIF-1α levels (% over control)
PD184161
PD98059
**
***
***
0 20 40 60 80 100nM
0 10 20 30 40 50μM

Figure 1

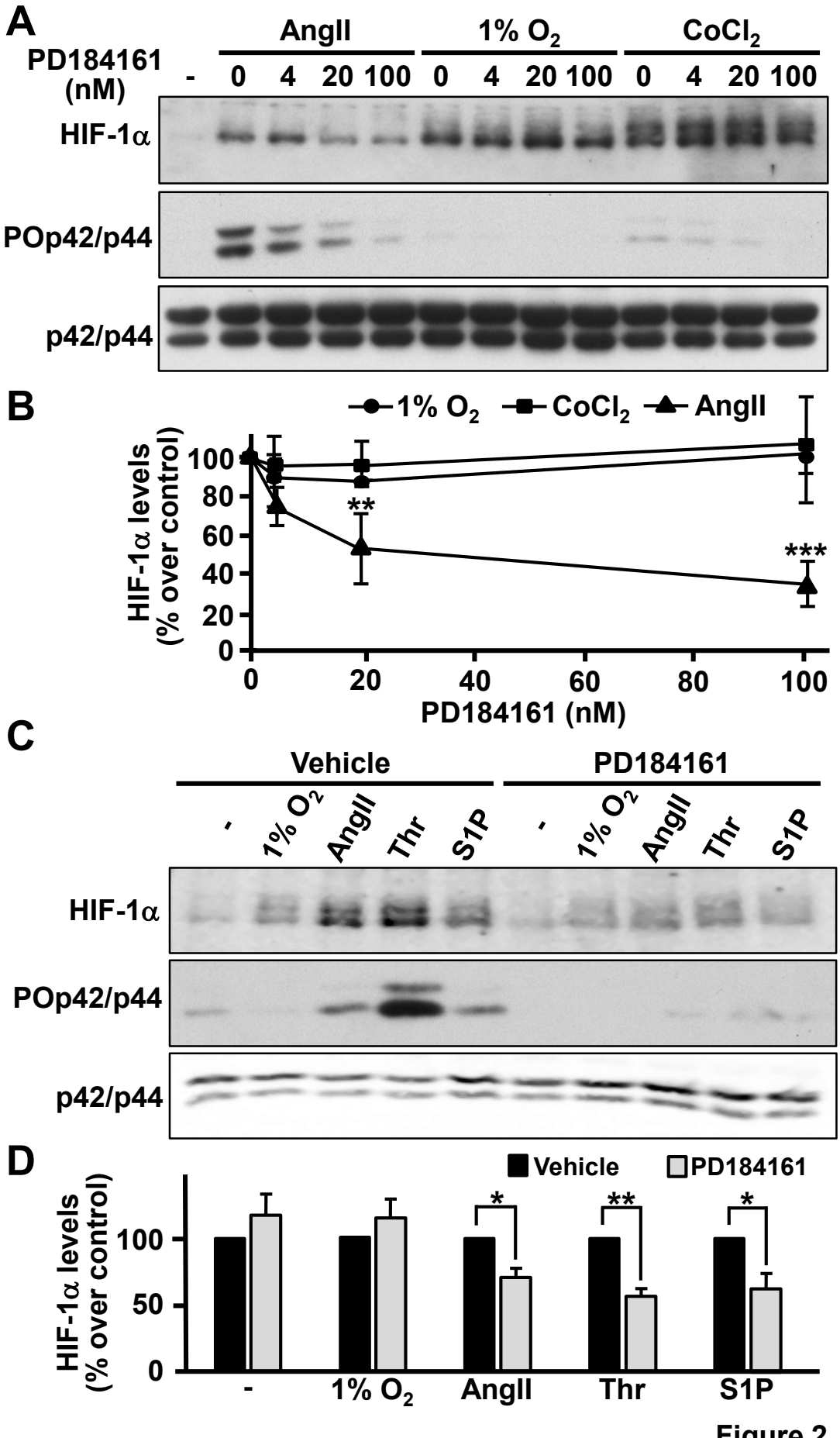
A
AngII
1% O2
CoCl2
PD184161 (nM)
-
0
4
20
100
HIF-1α
POp42/p44
p42/p44
B
1% O2
CoCl2
AngII
HIF-1α levels (% over control)
**
***
PD184161 (nM)
C
Vehicle
PD184161
-
1% O2
AngII
Thr
S1P
HIF-1α
POp42/p44
p42/p44
D
Vehicle
PD184161
HIF-1α levels (% over control)
*
**
*
-
1% O2
AngII
Thr
S1P

Figure 2

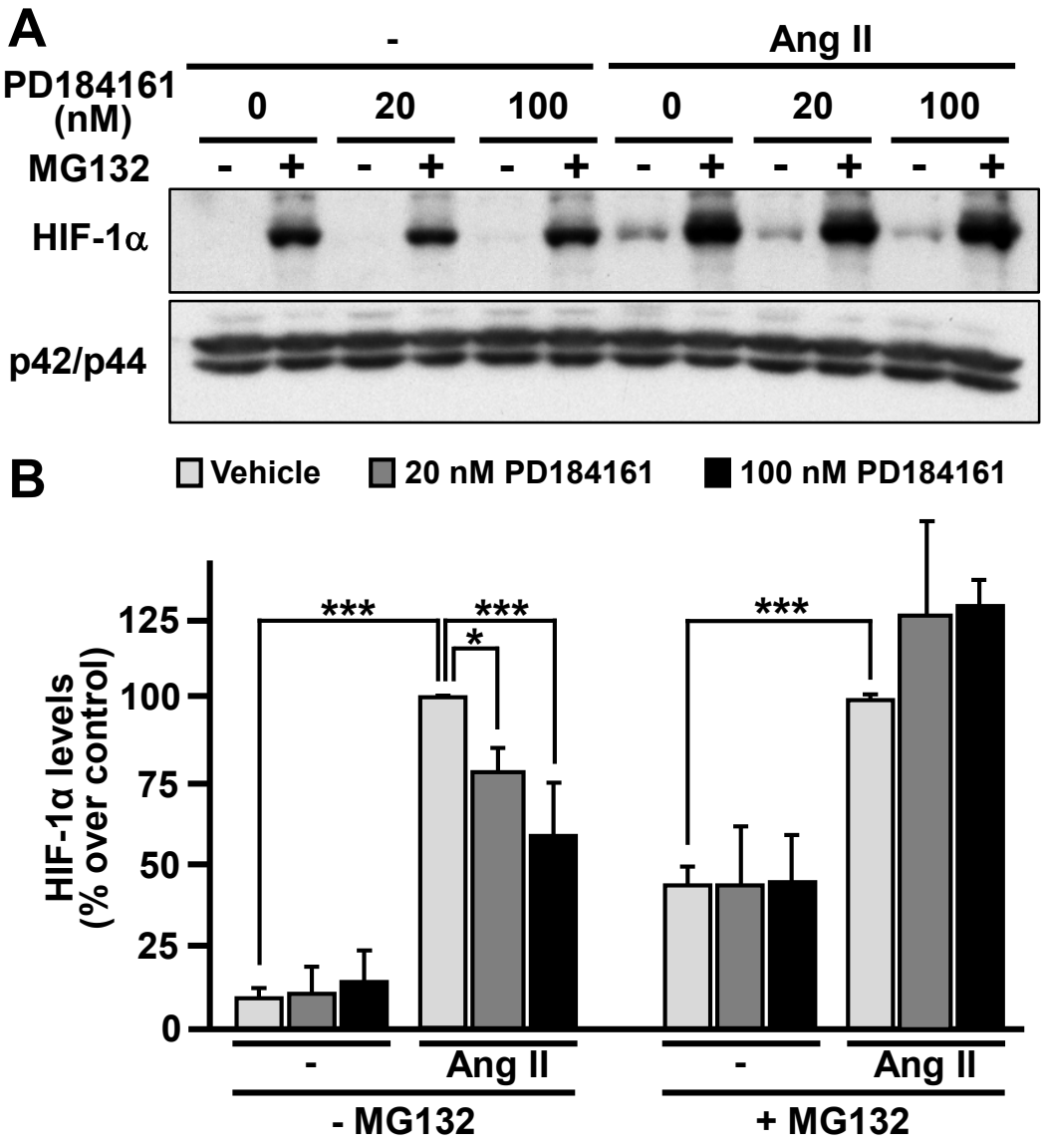
A
-
Ang II
PD184161 (nM)
0
20
100
0
20
100
MG132
- + - + - + - + - + - +
HIF-1α
p42/p44
B
Vehicle
20 nM PD184161
100 nM PD184161
HIF-1α levels (% over control)
125
100
75
50
25
0
***
***
*
***
-
Ang II
-
Ang II
- MG132
+ MG132

Figure 3

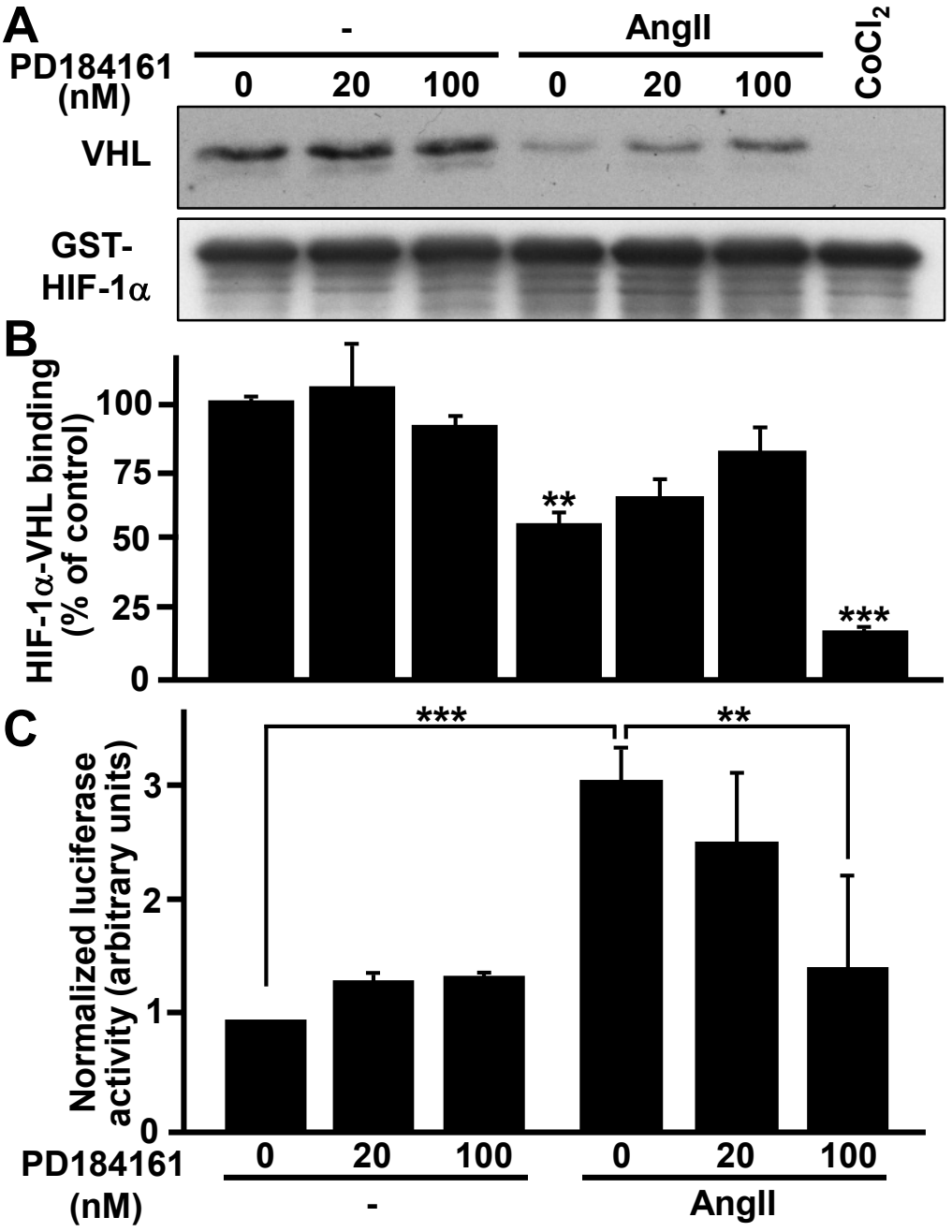
A
-
AngII
PD184161 (nM)
0
20
100
0
20
100
CoCl2
VHL
GST-HIF-1α
B
HIF-1α-VHL binding (% of control)
100
75
50
25
0
**
***
C
Normalized luciferase activity (arbitrary units)
3
2
1
0
***
**
PD184161 (nM)
0
20
100
0
20
100
-
AngII

Figure 4

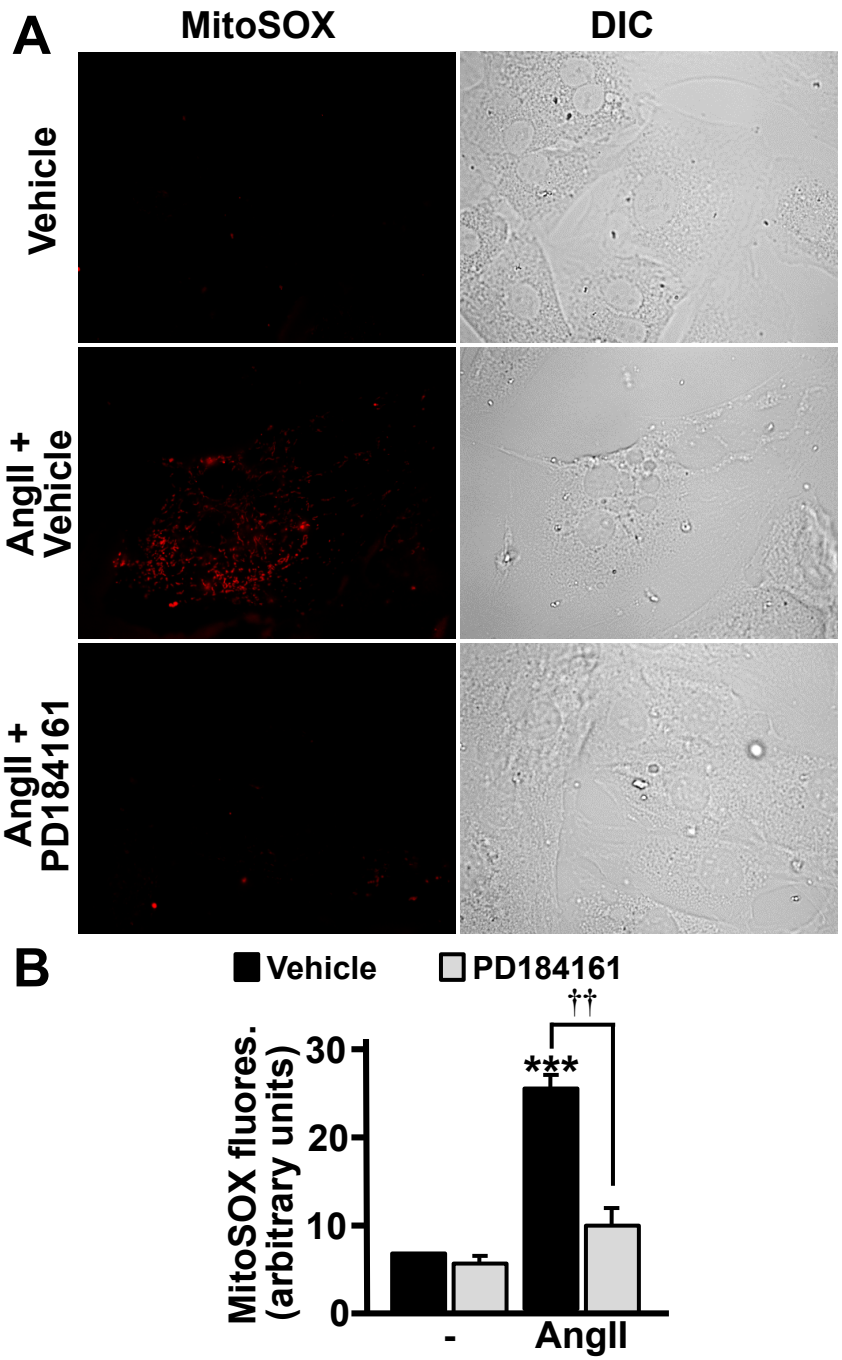
A
MitoSOX
DIC
Vehicle
AngII + Vehicle
AngII + PD184161
B
Vehicle
PD184161
MitoSOX fluores. (arbitrary units)
30
20
10
0
***
††
-
AngII

Figure 5

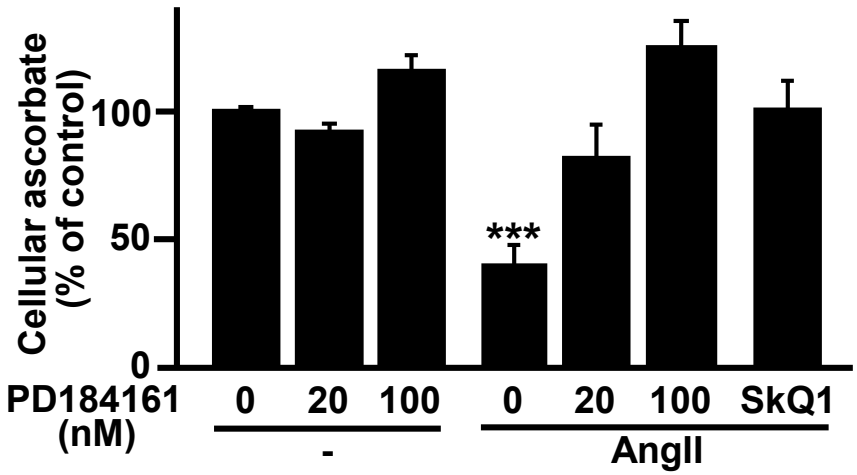
Cellular ascorbate
(% of control)
100
50
0
***
PD184161
(nM)
0
20
100
0
20
100
SkQ1
-
AngII

Figure 6

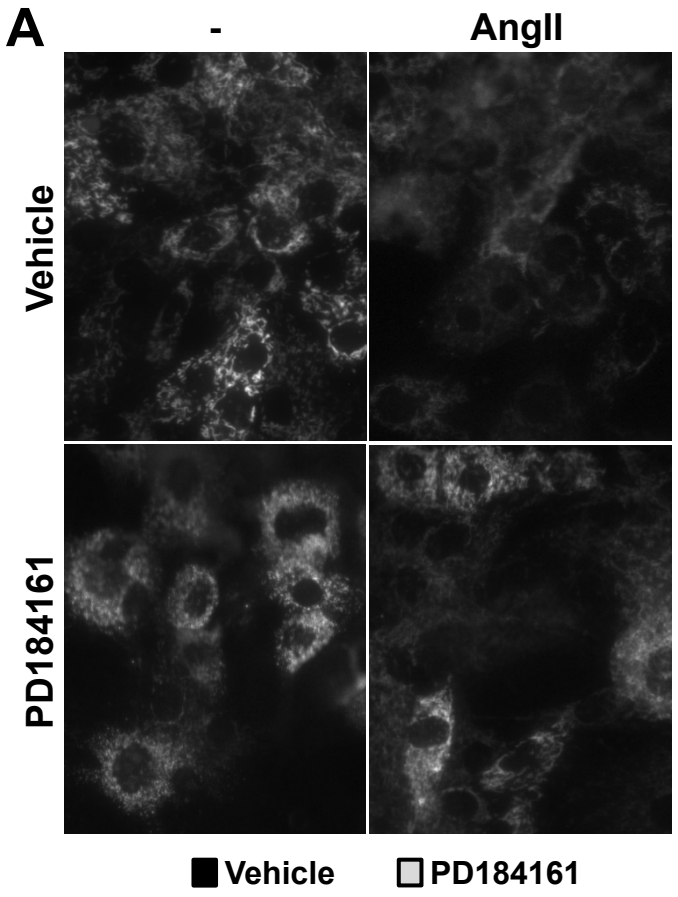
A
-
AngII
Vehicle
PD184161
Vehicle
PD184161
B
TMRM fluores. (arbitrary units)
40
30
20
10
0
**
-
AngII
C
Cellular ATP (% of control)
100
50
0
*
**
-
AngII

Figure 7