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Mechanism of Erythropoietin Regulation by Angiotensin II

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ACE, angiotensin II converting enzyme; Ang II, angiotensin II; AMPK, AMP-dependent protein kinase; AT_1R, angiotensin II type 1 receptor; AT_2R, angiotensin II type 2 receptor; CFU-E, colony forming unit-erythroid; CHO-K1, Chinese hamster ovary-K1 cells; EPO, erythropoietin; EPOR, EPO receptor; ERK, extracellular signal-regulated kinase; Egr-1, early growth response gene 1; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; Gab1, GRB2-associated binding protein 1; HIF, hypoxia-inducible factor; HRE, hypoxia response element; JNK/SAPK, c-Jun N-terminal kinases/stress-activated protein kinase; KO, knockout mouse; MEK, MAPK/ERK kinase; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PLC, phospholipase C; PKB, protein kinase B; rhEPO, recombinant human EPO; SHP1/2, c-Src homology-containing protein phosphatase; THM, Tsukuba hypertensive mouse; TM, transmembrane domain; VSMC, vascular smooth muscle cells.

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Abstract

Erythropoietin (EPO) is the primary regulator of red blood cell development. Although hypoxic regulation of EPO has been extensively studied, the mechanism(s) for basal regulation of EPO are not well understood. *In vivo* studies in healthy human volunteers and animal models indicated that angiotensin II (Ang II) and angiotensin converting enzyme inhibitors regulated blood EPO levels. In the current study we found that Ang II induced EPO expression *in situ* in murine kidney slices and in 786-O kidney cells in culture as determined by RT-PCR. We further investigated the signaling mechanism of Ang II regulation of EPO in 786-O cells. Pharmacological inhibitors of Ang II type 1 receptor (AT_1R) and extracellular signal-regulated kinase 1/2 (ERK1/2) suppressed Ang II transcriptional activation of EPO. Inhibitors of AT_2R or Src homology 2 domain-containing tyrosine phosphatase had no effect. Co-immunoprecipiation experiments demonstrated that p21Ras was constitutively bound to the AT_1R; this association was increased by Ang II but was reduced by the AT_1R inhibitor telmisartan. Transmembrane domain (TM) 2 of AT_1R is important for G protein-dependent ERK1/2 activation, and mutant D74E in TM2 blocked Ang II activation of ERK1/2. Ang II signaling induced the nuclear translocation of the Egr-1 transcription factor, and overexpression of dominant negative Egr-1 blocked EPO promoter activation by Ang II. These data identify a novel pathway for basal regulation of EPO via AT_1R-mediated Egr-1 activation by p21Ras-MEK-ERK1/2. Our current data suggest that Ang II, in addition to regulating blood volume and pressure, may be a master regulator of erythropoiesis.

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Introduction

Erythropoietin (EPO), a glycoprotein hormone, is the major regulator of red blood cell development (Wu et al., 1995). EPO controls erythropoiesis by modulating the maturational differentiation and proliferation of early erythroid progenitor cells, including burst-forming unit-erythroid (BFUe) and colongy-forming unit-erythroid (CFUe) cells, via the EPO receptor (EPOR)(Jelkmann, 2007; Wu et al., 1995). In mice, EPO or EPOR-deficiency is embryonically lethal due to severe anemia and other developmental abnormalities (Wu et al., 1995; Yu et al., 2002). EPO is primarily produced in adult kidney, but the liver is also source of EPO in the fetus and prenatal stages of development (Eckardt and Kurtz, 2005). Recombinant human EPO (rhEPO) is a clinically significant drug, utilized for the restoration of hemoglobin and red blood cell levels in anemia from a variety of causes, including chronic kidney disease, kidney failure, cancer or cancer therapy, AIDS, hepatitis C, congestive heart failure, and some surgical settings (Fisher, 2003; Jelkmann, 2007; Silverberg et al., 2010; Stickel et al., 2012). The worldwide therapeutic market for rhEPO for patient treatment was reported to be ~$6 billion USD/year between 2006 and 2010 (Lee et al., 2012).

Physiological anemia and oxygen depression are potent inducers of EPO expression (Ebert and Bunn, 1999; Fried, 2009). Under hypoxic conditions, hypoxia-inducible factors (HIFs) are the essential transcriptional regulators of the hypoxia response element (HRE) of the EPO gene promoter (Eckardt and Kurtz, 2005; Jelkmann, 2007). Evidence indicates that HIF-2α protein, and not HIF-1α, is required for hypoxia-induced expression of EPO in the kidney, in EPO-producing cultured renal cells, and in the fetal liver (Bunn, 2013; Rosenberger et al., 2002; Warnecke et al., 2004). In contrast, GATA transcription factors (GATA-1, -2 and -3) and NF-κB negatively regulate EPO gene transcription (Imagawa et al., 1997; La Ferla et al., 2002; Obara et al., 2008). Activation of GATA-2 and NF-κB in response to inflammatory cytokines interleukin-1β and tumor necrosis factor-α suppress EPO expression in cell culture (La Ferla et al., 2002). Other factors, including insulin, estrogen, androgens, and prostaglandin modulate EPO production, although the mechanisms of EPO regulation by these factors are unknown (Bunn, 2013; Ebert and Bunn, 1999; Hardee et al., 2006). Importantly, despite the clinical importance of EPO for patient treatment, the mechanism(s) for basal regulation of EPO expression in the kidney is not well understood.

Angiotensin II (Ang II), a peptide hormone, is a well-known regulator of blood pressure and blood volume homeostasis, and has been demonstrated to modulate the development of specific

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white blood cell lineages (Rodgers et al., 2000). Ang II receptors have been identified on hematopoietic progenitors, and findings indicate that Ang II can enhance the proliferation of specific hematopoietic lineages (Beyazit et al., 2007; Haznedaroglu and Ozturk, 2003; Rodgers et al., 2000). Importantly, Ang II has also been implicated in EPO regulation *in vivo*. Clinical studies of healthy human volunteers demonstrated that Ang II administration increased serum EPO concentration by ~35% or higher via the activation of the angiotensin II type 1 receptor (AT_1R)(Freudenthaler et al., 2000; Freudenthaler et al., 1999; Gossmann et al., 2001). Furthermore, the Ang II converting enzyme (ACE) inhibitors captopril and enalapril that block the maturation of Ang II from Ang I, significantly decreased plasma EPO levels, by as much as ~20-30% in another study of healthy human volunteers (Pratt et al., 1992). These findings suggest that Ang II regulates EPO production *in vivo* via receptor-dependent signaling, but the mechanism of this regulation has not been investigated.

Our laboratory recently demonstrated in wild type mice that the ACE inhibitor captopril transiently reduced plasma EPO levels to below 70%, resulting in a transient reduction of both red blood cells and reticulocytes (Barshishat-Kupper et al., 2011). Consistent with these observations, serum EPO levels and kidney EPO mRNA expression were demonstrated to be enhanced by the activation of the renin-angiotensin system in double transgenic Tsukuba hypertensive mice (THM) *in vivo* (Kato et al., 2005). Furthermore, EPO synthesis was also found to be reduced in THM/AT_1R-knockout mice (Kato et al., 2005). We hypothesized that Ang II receptor-dependent signaling is responsible for direct regulation of EPO under non-hypoxic conditions. In the present study, we demonstrate that Ang II induces EPO mRNA expression and increases EPO protein *in situ* and *in vitro*. We further demonstrate that AT_1R is required for Egr-1 activation via p21Ras-MEK-ERK signaling pathway for EPO expression. These novel findings suggest that Ang II and its receptor specific signaling pathways stimulate proliferation and differentiation of hematopoietic progenitor cells by means of efficient EPO production under non-hypoxic conditions.

Materials and methods

Cell lines and cell culture – Human renal 786-O cells were cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 (Invitrogen, Carlsbad, CA) medium containing 10% (v/v) heat-inactivated fetal bovine serum (Gemini Bioproducts, Woodland, CA) with penicillin (100 units/ml) and

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streptomycin (100 μg/ml) in a humidified 5 % CO_2 incubator at 37 ^{o}C. Cells were treated with Ang II in the presence of 5% serum.

Reagents and antibodies– Angiotensin II (Ang II) was purchased from Bachem, Inc. (Torrance, CA). Telmisartan and PD 123319 were purchased from Sigma-Aldrich (St. Louis, MO). SHP1/2 PTPase inhibitor, NSC-87877 was purchased from Calbiochem (San Diego, CA). U1026 was from Cell Signaling Technology (Danvers, MA). Antibodies against phospho-Thr202/Tyr204 ERK1/2 (#9101), and phospho-Ser217/221 MEK1/2 (#9152) were from Cell Signaling Technology (Danvers, MA). Antibodies against anti-ERK1 (sc-94), AT_1R (sc-1173, sc-579 and sc-31181 for co-immunoprecipitation), GFP (sc-9996), HA (sc-805 for immunoblotting and sc-805-G for co-immunoprecipitation), EPO (sc-7956), Egr-1 (sc-20689 for immunofluorescence), Egr-1 (sc-110 for immunoblotting), β-actin (sc-47778), Lamin B (sc-6217), α-tubulin (sc-8035), Gab1 (sc-9049 for western blots) and Gab1 (sc-6292 for co-immunoprecipitation) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-MEK1 and p21Ras antibody was from BD Biosciences (San Jose, CA). We used mouse monoclonal anti-WT1 antibody (clone 6F-H2) Millipore (Billerica, MA) and mouse monoclonal anti-HA antibody (12CA5) from Roche Applied Science (Indianapolis, IN).

Plasmids and transient transfection–We generated HA (hemagglutinin)-tagged AT_1R expression plasmid at *Xho*I and *Not*I with phCMV2. Two truncated forms of AT_1R (HA-tG306 and tL314; deletion mutant from 306th Glycine and 314th Leucine of AT_1R, respectively) were generated for tG306, forward-5'-GATCTCGAGATTCTCAACTCTTCTACTGAAGAT-GGTAT-3', reverse-5'-GTCGCGGCCGCTTACCCCAGAAAGCCATAAAAAAGAGGA-3'. The tL314, forward-5'-GATCTCGAGATTCTCAACTCTTCTACTGAAGATGGTAT-3', reverse-5'-GTCGCGGCCGCTTAGAGAAAATATCTTTTAAATTTTTTC-3'. The second transmembrane domain mutant (D74E) and the seventh transmembrane domain mutants (T287V, F293L and N295S) of AT_1R were previously described.(Yee et al., 2006) GFP-tagged human wild-type p21Ras (Ras) and dominant-negative mutant of p21Ras (Ras-S17N) were purchased from Addgene (Cambridge, MA). Wild type (wt) and dominant negative Egr-1 plasmids were previously described (Day et al., 2004). For transient overexpression, 786-O cells were grown to 70% confluence and

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transfected using Fugene 6 according to the manufacturer's recommendation (Roche Applied Science, Indianapolis, IN). After 48 h incubation, cell lysates were prepared (Kim and Day, 2012).

Ex vivo murine renal explants– Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Kidneys from 4 mice were harvested immediately after euthanasia and released from renal capsules. Animal handling procedures were performed in compliance with guidelines from the National Research Council and were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences (Bethesda, MD, USA). Peripheral sections containing the cortex were cut into 1-2 mm slices using a McIlwain Tissue chopper (GeneQ Inc., Quebec, Canada) under a Laminar flow hood. Slices were pooled and randomly divided into 2 × 12 well tissue culture plates (3-6 slices per well) containing Dulbecco's Modified Eagle Media/F-12 supplemented with 10% FBS. Slices were incubated for 30 min at 37 °C in a humidified cell culture incubator prior to treatments. Ang II treatments were performed in the presence of 10% serum.

Quantitative real-time PCR (qRT-PCR) – Total RNA was isolated from mouse kidney slices or 786-O cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Genomic DNA was removed using RNasefree DNase (Qiagen). RNA was quantified spectroscopically (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE), and integrity was assessed by capillary electrophoresis (Experion, Bio-Rad, Hercules, CA). Murine kidney RNA (0.5 μg) and 786-O cells RNA (1 μg) were subjected to reverse transcription with GeneAmp RNA PCR kit, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). PCR was performed in triplicates using SybrGreen PCR master mix (Applied Biosystems, Foster City, CA). Primers for qRT-PCR were designed using the ProbeFinder software, version 2.35 (Roche Applied Sciences, Indianapolis, IN). Primer pairs for murine EPO gene expression were: mEPO, forward-5'-TCTGCGACAGTCGAGTTCTG-3' and reverse-5'-CTTCTGCACAACCCATCGT-3'. Human EPO gene expression: hEPO, forward-5'-TCCCAGACACCAAAGTTAATTTCTA-3' and reverse-5'-CCCTGCCAGACTTCTACGG-3'. As internal control, mRNA levels of α-tubulin were determined. For quantification, the comparative threshold cycle method was used to assess relative changes in mRNA levels between the untreated and the drug-treated samples.

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Co-immunoprecipitation and immunoblotting assay – 786-O cells were grown to 80% confluence prior to treatment. Where noted, inhibitors (NSC-87877 or U0126) were pretreated for 1 h prior to Ang II treatment, followed by preparation of whole cell lysates (Kim and Day, 2012). For western blotting of EPO from kidney slices, the same lysis buffer was used. Co-immunoprecipitation and immunoblotting were performed as described previously (Kim and Day, 2012; Kim et al., 2011).

Luciferase assay – The luciferase-linked human EPO plasmid, pEPO (-282 bp to -7 bp), was constructed in pGL3-basic vector (Promega, Madison, WI). The oligonucleotides primers used for cloning the EPO promoter were: forward-5’-CGTGCTAGCACGCACACATGCAGATAACAG-3’, reverse-5’ CGGAAGCTTGCCTGGCCGGGGTCCCTCAGC-3’; the promoter was cloned into the *Nhe*I-*Hind*III sites of pGL3. The construct was sequenced using a Big Dye sequencing kit (Invitrogen, Carlsbad, CA). To analyze reporter activation, 0.2 µg/well of the human EPO promoter gene plasmids were transfected into 786-O cells in a 24-well plate using the Fugene 6 reagent (Roche Applied Science, Indianapolis, IN). To control for transfection efficiency, *Renilla* luciferase gene expression plasmids were cotransfected (pRL-TK, 150 ng/well). Luciferase and *Renilla* were measured using a dual luciferase system (Promega, Madison, WI).

Immunofluorescence assay – Immunofluorescence analysis was performed as described previously (Kim et al., 2009). Anti-rabbit polyclonal Egr-1 antibody from Santa Cruz Biotechnology, Inc. (sc-20689) and the Alexa 594 donkey anti-rabbit IgG secondary antibody were used for immunohistochemistry (Invitrogen). DAPI stain was performed with ProLong Gold antifade reagent (Invitrogen).

Statistical analysis –Significant differences between the two groups were statistically determined by the Student's *t*-test. For three or more groups, statistical analysis was performed using one-way ANOVA, followed by the Bonferroni post-analysis, as appropriate. *P* values were calculated by means ± standard deviations (SD), with $p < 0.05$ considered statistically significant difference.

Results

EPO gene is transcriptionally activated by Ang II ex vivo and in vitro. We first investigated Ang II regulation of EPO gene expression in murine kidney slices cultured *ex vivo*. Kidney slices were

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cultured for 30 min prior to treatment with Ang II for 4 h. Total RNA was isolated and EPO mRNA levels were measured using qRT-PCR (Fig. 1A). $CoCl_2$ was included as positive control for the induction of EPO expression (Fig. 1A). Data indicate that 100 μM Ang II induced EPO gene expression within 4 h. A time course revealed that Ang II treatment increased EPO mRNA expression within 2 h, maximal at 4 h (Fig. 1B). We found that EPO protein levels were also increased in kidney slice cultures in response to treatment with Ang II (Fig. 1C). Interestingly, an increase in EPO protein was observed as early as 1 h, suggesting that a non-transcriptional mechanism for EPO regulation may also be involved in whole tissue. We next investigated Ang II regulation of EPO in the 786-O cells, a human kidney adenocarcinoma cell line. In 786-O cells, 10 μM Ang II activated EPO mRNA expression ~2-fold within 6 h, and 50 μM Ang II activated expression of EPO gene by ~4.5-fold within 6 h (Fig. 1D). EPO protein was increased ~1.6-fold within 12 h (Fig. 1 E).

Ang II-induced activation of EPO requires AT_1R and the MEK1/2-ERK1/2 signaling pathway. The biological actions of Ang II are mediated primarily by two specific receptors, angiotensin II type 1 (AT_1R) and type 2 (AT_2R) receptors (Mogi et al., 2007). Both AT_1R and AT_2R belong to the seven transmembrane G protein-coupled receptor (GPCR) family, and the activation of AT_1R or AT_2R downstream signaling by Ang II has been demonstrated to be cell type- and tissue type-specific (Kim and Day, 2012; Lee et al., 2010; Mogi et al., 2007). We investigated the Ang II receptor subtype required for regulation of EPO in 786-O cells. Using qRT-PCR, we found that Ang II-induced transcriptional activation of EPO was inhibited by the AT_1R inhibitor, telmisartan. In contrast, Ang II-induced EPO levels were further increased ~1.6-fold by the AT_2R specific blocker, PD 123319, compared to Ang II alone (Fig. 2A). AT_1R and AT_2R have been demonstrated in some cell types to activate opposing signal transduction pathways, thus the inhibition of one Ang II receptor in some cases augments signaling by the alternate receptor, consistent with our results.

AT_1R has been demonstrated to signal through a variety of downstream molecules, including GTP-binding proteins, Src-homology 2 domain-containing tyrosine phosphatases (SHPs), and ERK1/2 MAPK (Day et al., 1999; Godeny et al., 2007; Olson et al., 2008). We examined the activation of EPO gene expression in the presence of inhibitors of kinase and phosphatase pathways. EPO mRNA was slightly reduced by the MEK1/2 inhibitor U0126 (Fig. 2A). Our laboratory previously demonstrated that SHP1/2 activation is important for Ang II signaling in

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primary endothelial cells (Lee et al., 2010). However, the SHP1/2 inhibitor NSC-87877 did not reduce Ang II-induced EPO mRNA (Fig. 2B). Ang II-induced EPO protein expression was blocked by telmisartan and U0126, indicating roles for AT_1R and MEK in EPO protein regulation (Fig. 2C).

The MAPK signaling pathway is an important regulator of gene regulation and activation (Chang and Karin, 2001), and is activated downstream of AT_1R in Ang II regulation of platelet-derived growth factor and plasminogen activator inhibitor-1 gene expression (Chen and Feener, 2004; Day et al., 1999). We investigated MEK1/2-ERK1/2 activation by Ang II in 786-O cells. Phospho-MEK1/2 (Ser217/221) was increased ~2-fold at 15 min after Ang II treatment, and was blocked by the presence of telmisartan (Fig. 2D). Phospho-ERK1/2 (Thr202/Tyr204) was induced within 15 min of Ang II treatment, and was significantly blocked by U0126 (Fig. 2E). Phosphorylation of ERK1/2 was not affected by NSC-87877 (Fig. 2F). We did not observe increased phosphorylation of a number of other kinases (protein kinase B (PKB/Akt), AMP-dependent protein kinase (AMPK), p38 MAPK and c-Jun N-terminal kinase/ stress-activated protein kinase (JNK/SAPK); data not shown), pathways demonstrated to be activated by Ang II in other cell types. Together our data suggest that MEK1/2 is required for ERK1/2 activation and for EPO regulation.

p21Ras is a primary upstream regulator of Ang II-induced activation of ERK1/2 and Ang II regulates AT_1R-p21Ras interaction. Both heterotrimeric G proteins and the small monomeric G protein Ras have been demonstrated to be activated by GPCRs (Dong and Wu, 2013; Marty and Ye, 2010). We investigated the potential role of p21Ras activation by AT_1R in 786-O cells. Either wild-type p21Ras (Ras) or a dominant-negative mutant of p21Ras (Ras-S17N) were expressed in 786-O cells for 48 h prior to treatment with Ang II. Ang II-induced phosphorylation of ERK1/2 was increased in the presence of Ras, but reduced >40 % in cells expressing Ras-S17N (Fig. 3A). Consistent with this, EPO protein levels were increased by Ang II in cells transfected with control vector or in cell expressing wild type Ras, but EPO was not induced by Ang II in cells expressing Ras-S17N (Fig. 3A).

Ang II was demonstrated to induce a direct association between AT_1R and p21Ras for MAPK activation in neurons (Yang et al., 1996). We investigated the association of endogenous AT_1R with p21Ras in 786-O cells. The interaction between AT_1R and p21Ras was confirmed by immunoprecipitation using an antibody against the C-terminal of AT_1R (Fig. 3B). Interestingly, the

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association between p21Ras and AT_1R was increased by Ang II within 5 min and was completely inhibited by telmisartan (Fig. 3B). The interaction was confirmed using exogenous expression of GFP-tagged p21Ras and HA-tagged AT_1R. Treatment of transfected cells with Ang II increased the association between HA-AT_1R and GFP-p21Ras, but treatment with telmisartan reduced this interaction (Fig. 3C), consistent with our findings with endogenous Ras association. We examined the binding of GFP-p21Ras with HA-tagged wild type AT_1R (WT- AT_1R) or two mutants of AT_1R truncated in the cytoplasmic C-terminal domain (one truncated after G306 (tG306) and the other truncated after L314 (tL314)). Previous studies have shown that truncation of AT1R at regions from 302 and above preserve Ang II binding in cellular expression systems but result in loss of other downstream signaling (Becker et al., 2004; Seta et al., 2002). Co-immunoprecipitation of p21Ras was reduced (>60 %) by both truncation mutants (Fig. 3D). This suggests that the C-terminus of AT_1R contains some but not all of the binding requirements for Ras interactions.

Gab1 is associated with AT1R in 786-O cells. A variety of adaptor proteins have been demonstrated to transduce the activation of p21Ras by GPCRs. GRB2-associated binding protein 1 (Gab1) activates the Ras/ERK/MAPK pathway in response to a number of ligands.(Cai et al., 2002; Montagner et al., 2005) We investigated the association of endogenous Gab1 with AT_1R and regulation by Ang II. Reciprocal co-immunoprecipitation with anti-AT_1R (C-terminal cytoplasmic 306-359 regions) or Gab1 antibodies for endogenous AT_1R and Gab1 interaction indicated that Gab1 was constitutively associated with AT_1R in 786-O cells (Fig. 4). Unlike the AT_1R-p21Ras interaction, Ang II and telmisartan had no effect on Gab1 association (data not shown).

The D74 mutant in the TM2 of AT_1R abrogates ERK1/2 activation by Ang II. Our data indicated that MEK1/2 was upstream of ERK1/2 activation and EPO regulation in 786-O cells. We wished to identify regions of AT_1R required for ERK1/2 activation in 786-O cells. Previous mutational studies demonstrated that specific amino acids in transmembrane domains two (TM2) and seven (TM7) are important for either the activated conformation of AT_1R for ERK activation and/or for G protein/PLC/PKC signal transduction.(Yee et al., 2006) D74 (TM2) was demonstrated to be crucial for G protein-dependent signaling but not for G protein-independent activation of ERK1/2 by AT_1R, and this amino acid is highly conserved in among G protein-coupled receptors (Aplin et al., 2009; Bihoreau et al., 1993). We utilized ectopic expression of a number of AT_1R mutants to

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identify regions of the receptor required for ERK1/2 activation in 786-O cells. The cells were transfected with wild-type AT_1R (AT_1R), or a mutant of TM2 (D74E), or mutants of TM7 (T287V, F293L, or N295S) for 48 h (Yee et al., 2006). ERK activation was determined after Ang II stimulation for 15 min, and all transfected cells were analyzed for the levels of receptor expression. We observed that Ang II increased ERK1/2 phosphorylation by ~2-fold in cells overexpressing wild type AT_1R (Fig. 5A). Interestingly, the D74E mutant did not increase basal levels of ERK1/2 phosphorylation but did block ERK1/2 activation by Ang II (Fig. 5B).We observed increased basal activation of ERK1/2 in cells overexpressing T287V, F293L, and N295S (Fig. 5B). In cells transfected with F293L and N295S, Ang II still induced a significant increase in ERK1/2 phosphorylation, but not in cells expressing T287V. These data indicate that Ang II activation of ERK1/2 activation in 786-O cells was blocked by the D74E mutation in TM2.

Egr-1 transcription factor regulates activation of EPO gene. ERK-dependent early growth response gene 1 (Egr-1) transcription factor mediates Ang II regulation of platelet-derived growth factor A-chain and cylin D in vascular smooth muscle cells (VSMC) and in Chinese hamster ovary cells (CHO), respectively (Day et al., 1999; Guillemot et al., 2001). We examined the potential role of Egr-1 in EPO gene regulation in 786-O cells. Egr-1 accumulated in nucleus and was reduced in cytoplasm after 30 min of Ang II stimulation (Fig. 6A). Since the Wilms Tumor 1 (WT1) transcriptional factor belongs to the Egr-1 family of transcription factors (Little et al., 1992) and it has been reported that WT1 is a transcriptional activator of the EPO gene (Dame et al., 2006), we also tested the activation of endogenous WT1 by Ang II. In contrast with our findings for nuclear accumulation of Egr-1, WT1 was increased ~70% in cytoplasm after Ang II treatment (Fig. 6A). Immunohistochemistry data supported cell fractionation data indicating that Ang II-induced Egr-1 nuclear translocation (Fig. 6B).

We investigated the role of Egr-1 in Ang II regulation of EPO using a luciferase reporter plasmid containing a portion of the human EPO gene promoter, pEPO (-282 bp to -7 bp). Ang II increased the expression of pEPO ~2-fold (Fig. 6C). Overexpression of wild-type Egr-1 (WT-Egr-1) with the pEPO further enhanced an Ang II activation by ~1.5-fold (Fig. 6D). In contrast, Ang II activation of pEPO was inhibited to basal levels by co-transfection of dominant-negative Egr-1 (DN-Egr-1) (Fig. 6D). Taken together, these observations suggest that Egr-1 plays an important role in the Ang II-induced activation of EPO gene by direct bindings in the promoter region.

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Discussion

Erythropoietin has been extensively studied for its role in erythropoiesis, and increasing evidence has demonstrated that EPO also has critical biological functions in other cell types including neurons, endothelial cells, and epithelial cells from a variety of tissues (Brines and Cerami, 2006; Siren et al., 2001; Wang et al., 2009). Despite the expanding insights into the role of EPO in overall biological homeostasis, the mechanism of basal (non-hypoxic) regulation of EPO is not understood. Clinical and preclinical studies have provided evidence that EPO is regulated by Ang II levels in the plasma (Barshishat-Kupper et al., 2011; Freudenthaler et al., 1999; Pratt et al., 1992). In the current study, we demonstrate that Ang II signaling regulates transcriptional activation of EPO in kidney slice cultures and in renal 786-O cells. Moreover, Ang II-induced EPO regulation is mediated by Egr-1 transcription factor activation via AT_1R medicated p21Ras-ERK1/2 signaling in 786-O cells (Fig. 7). The role of Ang II in blood pressure and blood volume regulation has been well-recognized, and Ang II has more recently been demonstrated to modulate specific white blood cell lineage development (Beyazit et al., 2007; Haznedaroglu and Ozturk, 2003; Rodgers et al., 2000). Our current data provide a molecular mechanism for Ang II transcriptional regulation of EPO expression. Together these findings underscore the importance of Ang II as a master regulator of blood homeostasis, including erythropoiesis.

In 786-O cells we found that p21Ras was required for Ang II activation of ERK1/2 downstream of AT1R. The intracellular domains of GPCRs have been demonstrated to activate both monomeric small GTPases and heterotrimeric G proteins that transduce numerous signaling cascades (Dong and Wu, 2013; Neves et al., 2002; Waters et al., 2004). GPCRs have been shown to broadly regulate heterotrimeric G proteins in the physiological actions including metabolic homeostasis, gene transcription, and cell migration (Neves et al., 2002). Interactions between GPCR and small GTPases were also demonstrated to be important for intracellular trafficking of GPCRs and for the regulation of phospholipase D and ERK1/2 (Dong and Wu, 2013; Esseltine et al., 2011).

The structural determinants for AT_1R activation of the ERK1/2 signaling pathway remain controversial (Belcheva and Coscia, 2002; Yee et al., 2006). AT_1R undergoes a conformational change upon ligand and agonist binding, resulting in the activation of both G protein-dependent and -independent signaling. Furthermore, there appear to be variations in the signal transduction by mutants of AT_1R in different cellular backgrounds (Doan et al., 2001). p21Ras association with

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AT_1R was increased in the presence of Ang II but blocked in the presence of the AT_1R antagonist telmisartan, suggesting that the activation state of the receptor is important for Ras binding. Our data indicate that the D74E mutant of TM2 of AT_1R inhibited Ang II-induced activation of ERK1/2 in adult kidney 786-O cells without elevating the basal level of ERK1/2 phosphorylation. Findings from other laboratories indicated that the D74E mutation did not significantly alter the affinity of AT_1R for Ang II, but this mutation abrogated heterotrimeric G protein activation (Bihoreau et al., 1993). In our studies, the T287V (TM7) mutant blocked Ang II-induced ERK1/2 activation, but the basal level of ERK1/2 phosphorylation was increased significantly in cells transfected with this mutant. In previous studies, the T287V mutant was demonstrated to have normal G protein and ERK1/2 activation in COS-1 cells, without observed increased basal levels of activity (Yee et al., 2006). We hypothesize that the difference between the previously observed results in COS-1 cells may be due to a cell-type specific difference from the 786-O cells. Given the increased background in basal ERK1/2 activation, it is difficult to determine whether failure of Ang II to further induce ERK1/2 is due to abrogation of a downstream signaling interaction, or if it is due to constitutive activation of the receptor that is not further activated by Ang II. Together, our data regarding the effect of D74E mutant and the requirement of Ras suggest that a G protein-dependent pathway is the primary mechanism by which Ang II activates ERK1/2 in 786-O cells through AT_1R.

Our structural studies also demonstrated that the AT_1R-p21Ras interaction was reduced by truncation mutants, tG306 and tL314. This finding suggests that the c-terminal region of AT_1R may be involved in p21Ras binding, but does not completely encompass the binding elements. Thomas *et al*. showed that truncation of the last 45 amino acids of AT_1R (tL314) affected Ang II-induced AT_1R endocytosis, but had little effect on the G protein coupling in CHO-K1 cells (Thomas et al., 1995). Another study indicated that the third intracellular cytoplasmic loop domain of AT_1R had regulatory functions for Ras-dependent ERK activation in human embryonic kidney 293 cells (Haendeler et al., 2000). Interestingly, our data also show for the first time that AT_1R directly interacts with the Gab1 docking protein. This interaction was constitutive and not modulated by Ang II or telmisartan stimulation (data not shown). Overall, we interpret our data to indicate that AT_1R and p21Ras interaction requires the active AT_1R conformation, whereas the interaction between AT_1R and Gab1 is constitutive and does not require Ang II-induced AT_1R conformational activation.

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Our results in 786-O cells suggest that p21Ras-ERK1/2-Egr-1signaling increase EPO expression through gene transcription. Interestingly, in kidney slice cultures, Ang II increased EPO protein levels within 1 h, prior to the observed increase in EPO mRNA, suggesting that additional posttranscriptional mechanisms may also be involved. It is possible that in intact kidney tissue the regulation of EPO by Ang II is more complex, including multiple mechanisms of activation. Further research is required to identify additional mechanisms of EPO regulation by Ang II and other factors.

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Authorship contribution

Participated in research design: Kim, McCart, Mungunsukh, Rohrich, Yee, and Day.

Conducted experiments: Kim, McCart, Mungunsukh, and Rohrich.

Performed data analysis: Kim, McCart, Mungunsukh, Yee, and Day.

Wrote or contributed to the writing of the manuscript: Kim, McCart, Mungunsukh, Roehrich, Yee, and Day.

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Figure legends

Figure 1. Ang II induces both mRNA and protein levels of EPO *in vivo* and *in vitro*. (A,B) Ang II-induced transcriptional EPO gene expression in murine kidney slices. Kidney slices were incubated in culture medium for 30 min, and then treated with Ang II in the presence of serum. EPO mRNA levels were determined by RT-PCR. The α-tubulin was determined by RT-PCR for normalization. Cobalt (II) chloride ($CoCl_2$, 500 μM) was used as positive control. (A) Kidney slices were treated with the indicated concentrations of Ang II. Bar graphs show the fold change of EPO expression quantified as the ratios to the control (Cont). The data show means ± SD. * indicates p <0.05 *versus* the respective control, n=3. (B) Time-course of Ang II (100 μM)-induced mRNA levels of EPO in murine kidney tissue. Total mRNA was isolated after 1, 2, 4, 6 and 16 h. Bar graphs show the fold change of EPO expression and was quantifying as the ratio to the control (Cont). The data show means ± SD. * p <0.05 versus the respective control, n=3. (C) Protein levels of EPO were determined at the indicated time points following Ang II (100 μM) treatment of kidney slices. Whole cell lysates were western blotted for EPO antibodies; the membrane was stripped and blotted for α-tubulin. Bar graphs indicate EPO protein levels normalized to α-tubulin. Control (Cont) levels were set to 1. Data show means ± SD, n=3. * indicates p <0.05 versus control. (D) Ang II-induced transcriptional levels of EPO expression in kidney 786-O cells. The cells were treated at various time points with 10 μM or 50 μM of Ang II. $CoCl_2$ (60 μM) was used as positive control to induce EPO. Total mRNA was isolated after 2, 4, 6, 12 or 24 h. RT-PCR was performed using human EPO primers. α-tubulin was determined by RT-PCR for normalization. Bar graphs show the fold change of EPO expression quantified as the ratio to the control (Cont). The data show

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means ± SD, *n*=3. * indicates *p* <0.05 versus control. (E) Protein levels of EPO were determined at the indicated time points following Ang II (50 μM) treatment of 786-O cells. Whole cell lysates were western blotted for EPO antibodies; the membrane was stripped and blotted for α-tubulin. Bar graphs indicate EPO protein levels normalized to α-tubulin. Control (Cont) levels were set to 1. Data show means ± SD, *n*=3. * indicates *p* <0.05 versus control.

Figure 2. Ang II-induced EPO expression is dependent on AT_1R and MEK-ERK signaling pathway. (A-G) 786-O cells were pretreated with inhibitors for 30 min (telmisartan and PD) or 60 min (NSC and U0126) before treatment with Ang II (50 μM). Inhibitors were: AT_1R inhibitor telmisartan (1 μM), AT_2R inhibitor PD 123319 (50 μM, PD), SHP1/2 PTPase inhibitor NSC-87877 (50 μM, NSC), or MEK1/2 inhibitor U0126 (50 μM). (A, B) Cells were treated with Ang II or $CoCl_2$ (60 μM, a positive control for EPO induction) for 6 h prior to purification of mRNA. qRT-PCR was performed for EPO and normalized to α-tubulin. (C-G) Cells were treated with Ang II for the indicated times with or without inhibitors. Whole cell lysates were obtained for western blotting. (C) Lysates were blotted for EPO; membranes were stripped and blotted for β-actin for normalization. EPO is indicated by an arrow. (D) Lysates were blotted for phosphorylated MEK1/2 (p-MEK1/2-Ser217/221). Membranes were stripped and blotted for total MEK1. (E) Lysates were blotted for phosphorylated MEK1/2 (p-MEK1/2-Ser217/221) and ERK1/2 (p-ERK1/2-Thr202/Tyr204). Membranes were stripped and blotted for total MEK1 or ERK1. (F) Lysates were blotted for phosphorylated ERK1/2 (p-ERK1/2-Thr202/Tyr204). The membrane was reblotted for total ERK1 as a loading control. Band densities were normalized to either β-actin or to the total amount of nonphosphorylated protein. All bar graphs indicate normalized levels of proteins compared with normalized controls. Data show means ± SD, *n*=3. * indicates *p* <0.05 versus control. † indicates *p* <0.05 versus Ang II treatment at the same time point.

Figure 3. Ang II regulates interactions between AT_1R and p21Ras. (A) 786-O cells, 70% confluent, were transfected with the GFP empty vector, or GFP-tagged wild-type p21Ras (Ras) or dominant negative p21Ras (Ras-S17N) for 48 h. Cells were treated with Ang II (50 μM) for 15 min. Whole cell lysates were western blotted for phosphorylated ERK1/2 (p-ERK1/2-Thr202/Tyr204) or

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EPO. Membranes were reblotted for ERK1, β-actin, or GFP as loading controls. Bar graphs indicate normalized band densities from controls. Data show means ± SD. * indicates $p < 0.05$ for Ang II treatment versus untreated control in the same transfection, † indicates $p < 0.05$ versus the same condition of cells transfected with wild-type p21Ras; $n = 3$. (B) 786-O cells were pretreated with 1 μM telmisartan for 30 min prior to the addition of 50 μM Ang II for 5 min. Endogenous AT_1R was immunoprecipitated using the anti-AT_1R antibody followed by immunoblot with the anti-p21Ras antibody and AT_1R antibody for loading control. Individual antibodies of AT_1R were immunoprecipitated for the comparison of interaction patterns with p21Ras. Note that AT_1R in the input lane is difficult to detect without immunoprecipitation because endogenous expression is low; endogenous AT_1R expression is increased by Ang II treatment. (C) 786-O cells were grown to 70% confluence before transfection with HA-tagged wild-type AT_1R and GFP-tagged p21Ras for 48 h. Cells were treated with telmisartan (1 μM) for 30 min prior to stimulation with Ang II (50 μM) for 5 min. (D) 786-O cells were grown to 70% confluence and transfected with HA-empty vector, HA-tagged wild-type AT_1R (WT-AT_1R), or truncated HA-tagged AT_1R mutants (tG306 and tL314) and cotransfected with GFP-tagged p21Ras (GFP-p21Ras) for 48 h. HA-AT_1R was immunoprecipitated followed by immunoblotting for GFP-p21Ras.

Figure 4. AT_1R associates with Gab1. Endogenous AT_1R was immunoprecipitated followed by immunoblotting for Gab1 or for AT_1R as a loading control (left panel). Endogenous Gab1 was immunoprecipitated followed by immunoblotting for AT_1R or for Gab1as a loading control (right panel).

Figure 5. D74E mutant of the TM2 of AT_1R is involved in ERK activation by Ang II. 786-O cells were grown to 70% confluence before transfected with wild-type AT_1R (AT_1R) (A); or empty vector, AT_1R, T287V, D74E, F293L, or N295S (B) for 48 h, subsequently treated with Ang II (50 μM) for an additional 15 min prior to harvesting cell lysates. Whole cell lysates were western blotted for phosphorylated ERK1/2 (p-ERK1/2-Thr202/Tyr204). The membrane was stripped and reblotted with total ERK1 as a loading control. Bar graphs indicate normalized band densities from basal nonphosphorylated MAPK to phosphorylated MAPK (activated form). Data show means ± SD. * indicates $p < 0.05$ versus the basal control of cells transfected with wt AT_1R; † indicates $p < 0.05$ for Ang II treatment versus untreated control in the same transfection, n=3.

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Figure 6. Nuclear translocation and binding of Egr-1 to the EPO promoter. (A) 786-O cells were treated with 50 μM Ang II for 30 min and nuclear and cytoplasmic fractions were prepared for western blotting for Erg-1 and WT1. Lamin B and β-actin were loading controls for nuclear and cytoplasmic protein, respectively. Bar graphs indicate normalized band densities. Data show means ± SD. * indicates p <0.05 versus the respective control, n=3. (B) 786-O cells were treated with 50 μM Ang II for 30 min followed by immunohistochemistry for Egr-1. Fluorescent microscopy was performed on an Olympus BX61 fluorescence microscope (magnification ×20). Images were obtained using a Retiga EXi Aqua CCD camera (numerical aperture 0.70) from QImaging (Surrey, British Columbia, Canada) with iVistion-Mac^{TM} software (BioVision Technologies, Exton, PA). Scale bars indicate 50 μm. (C) 786-O cells were transfected with pGL3-basic vector (pGL) or pEPO. After 6 h, cells were treated +/- Ang II (50 μM) for 24 h. Bar graphs indicate the relative induction of luciferase normalized to *Renilla*. Graphs show means ± SD. * indicates p <0.05 for Ang II treatment versus the respective control, n=3. (D) 786-O cells were transfected with empty vector, wild-type Egr-1 (WT-Egr-1), or dominant-negative mutant of Egr-1 (DN-Egr-1), and cotransfected with pEPO and pRL-TK. After 6 h, cells were treated +/- Ang II (50 μM) for 24 h. Bar graphs show the relative induction of EPO promoter-driven luciferase activity normalized to *Renilla* activity. Graphs show means ± SD. * indicates p <0.05 for Ang II treatment versus the respective transfection control, n=3.

Figure 7. Schematic model of Ang II-induced regulation of EPO in 786-O cells. Proposed mechanism for EPO regulation upon Ang II stimulation via physiological interaction between AT_1R and p21Ras. Ang II binding to AT_1R transduces downstream signaling including the p21Ras-MEK1/2-ERK1/2 cascades and subsequent activation of Egr-1 for transcriptional activation of EPO in 786-O cells.

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Fig. 1

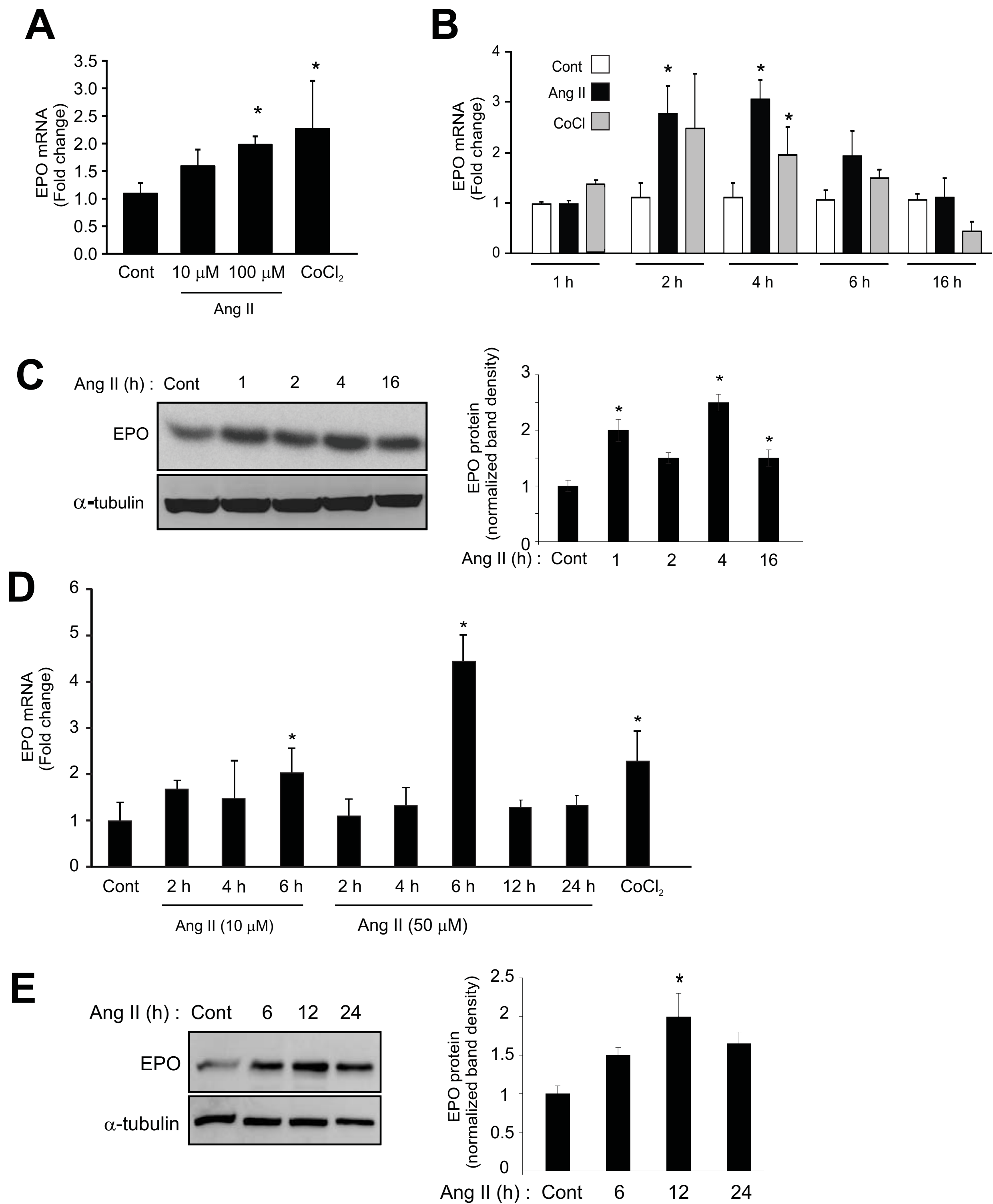

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Fig. 2

A

EPO mRNA (Fold change)

Cont | Ang II | Ang II + Telmisartan | Ang II + PD | $CoCl_2$

B

EPO mRNA (Fo d change)

Cont | Ang II | Ang II + NSC | Ang II + U0126 | $CoCl_2$

C

Telmisartan : +
U0126 : +
Ang II (h) : Cont 12 24 12 12

EPO →

β actin

EPO protein (normalized band density)

Telmisartan : - - - + -
U0126 : - - - - +
Ang II (h) : - 12 24 12 12

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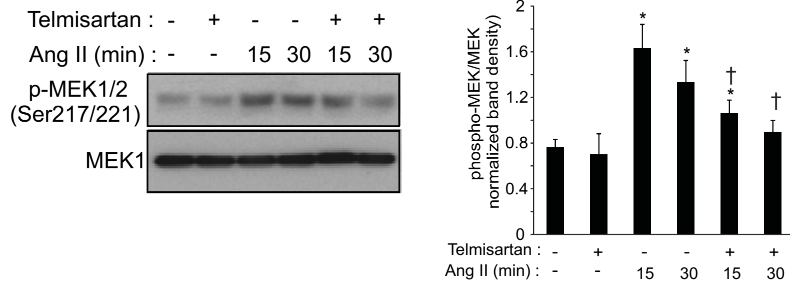
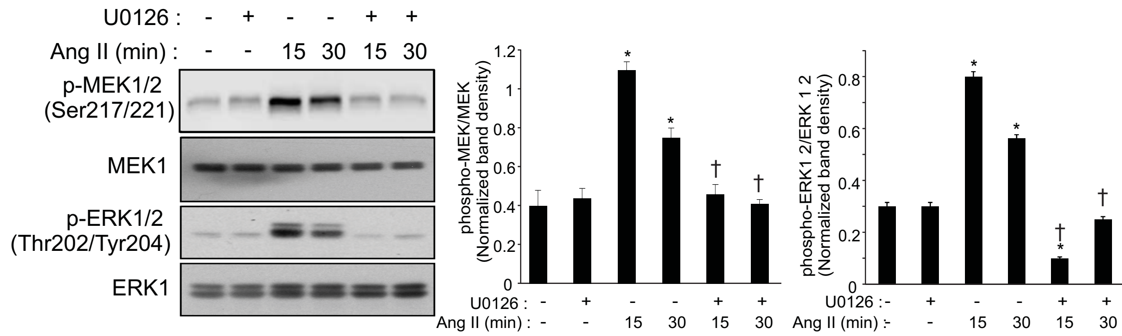
Fig. 2





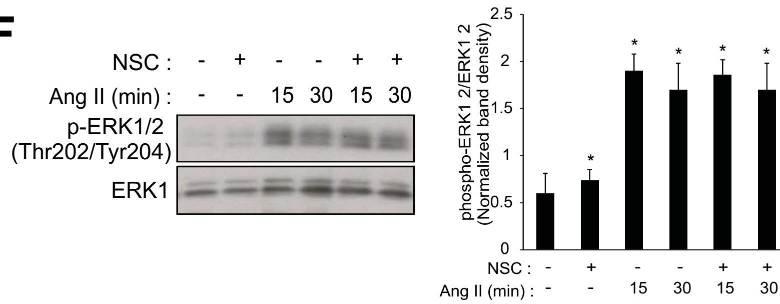


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Fig. 3

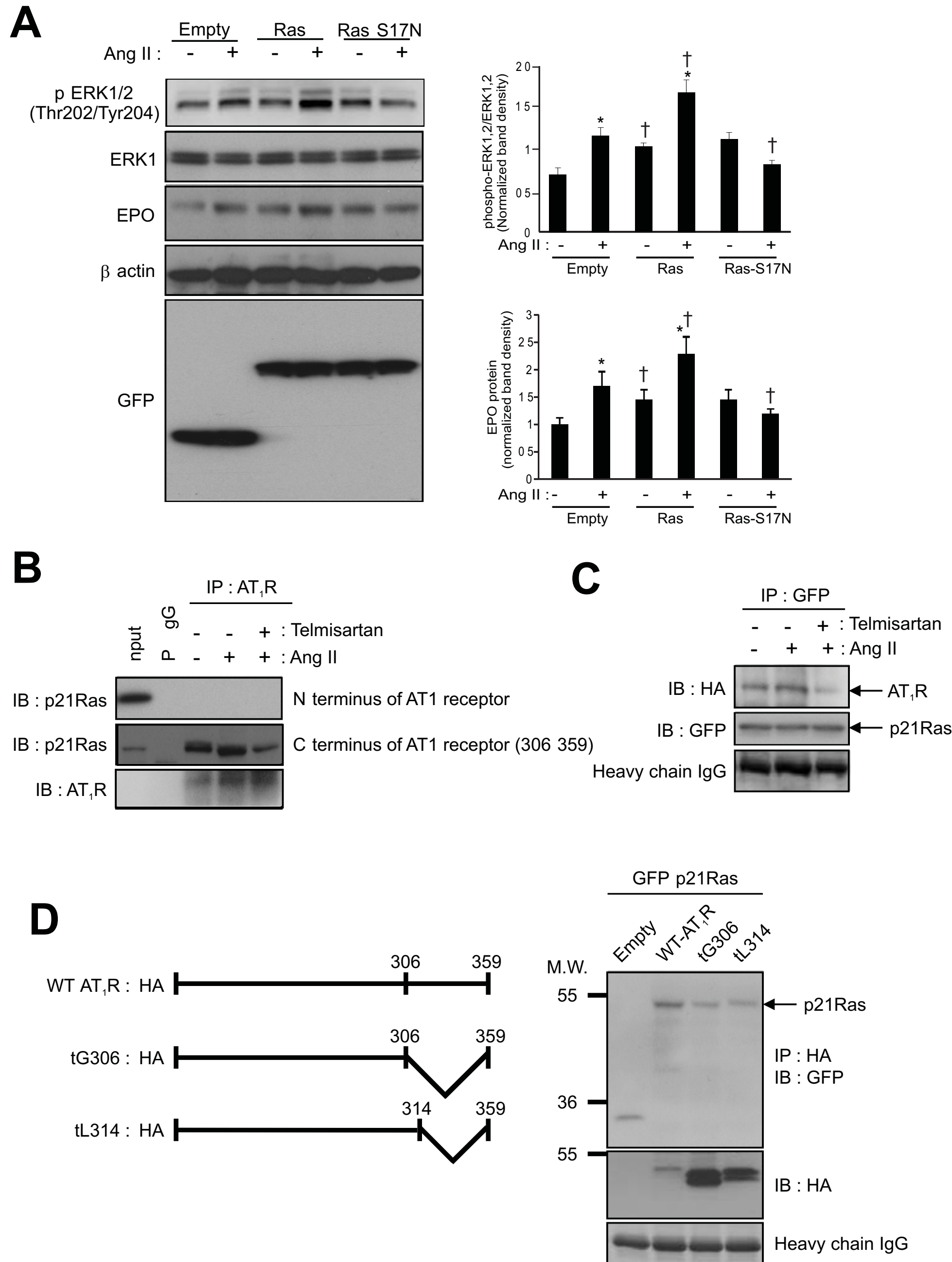

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Fig. 4

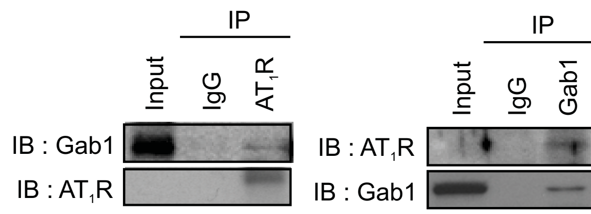

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Fig. 5

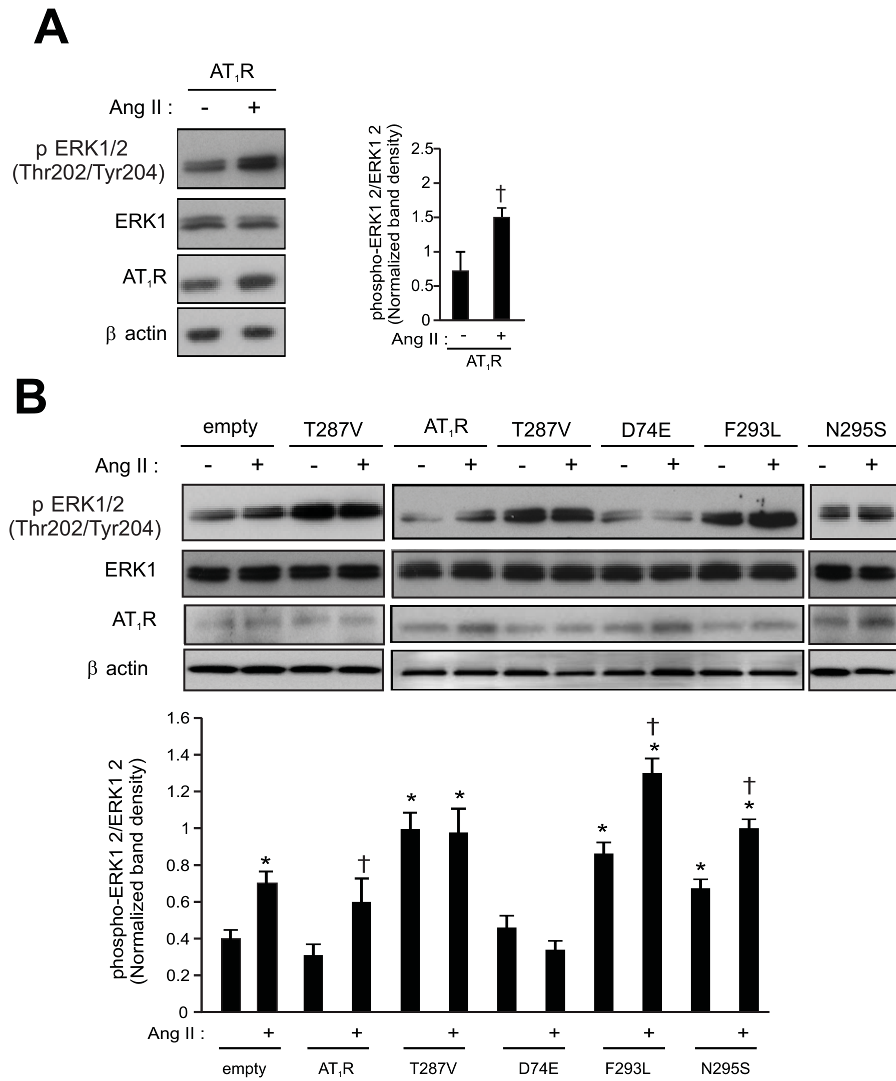

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Fig. 6

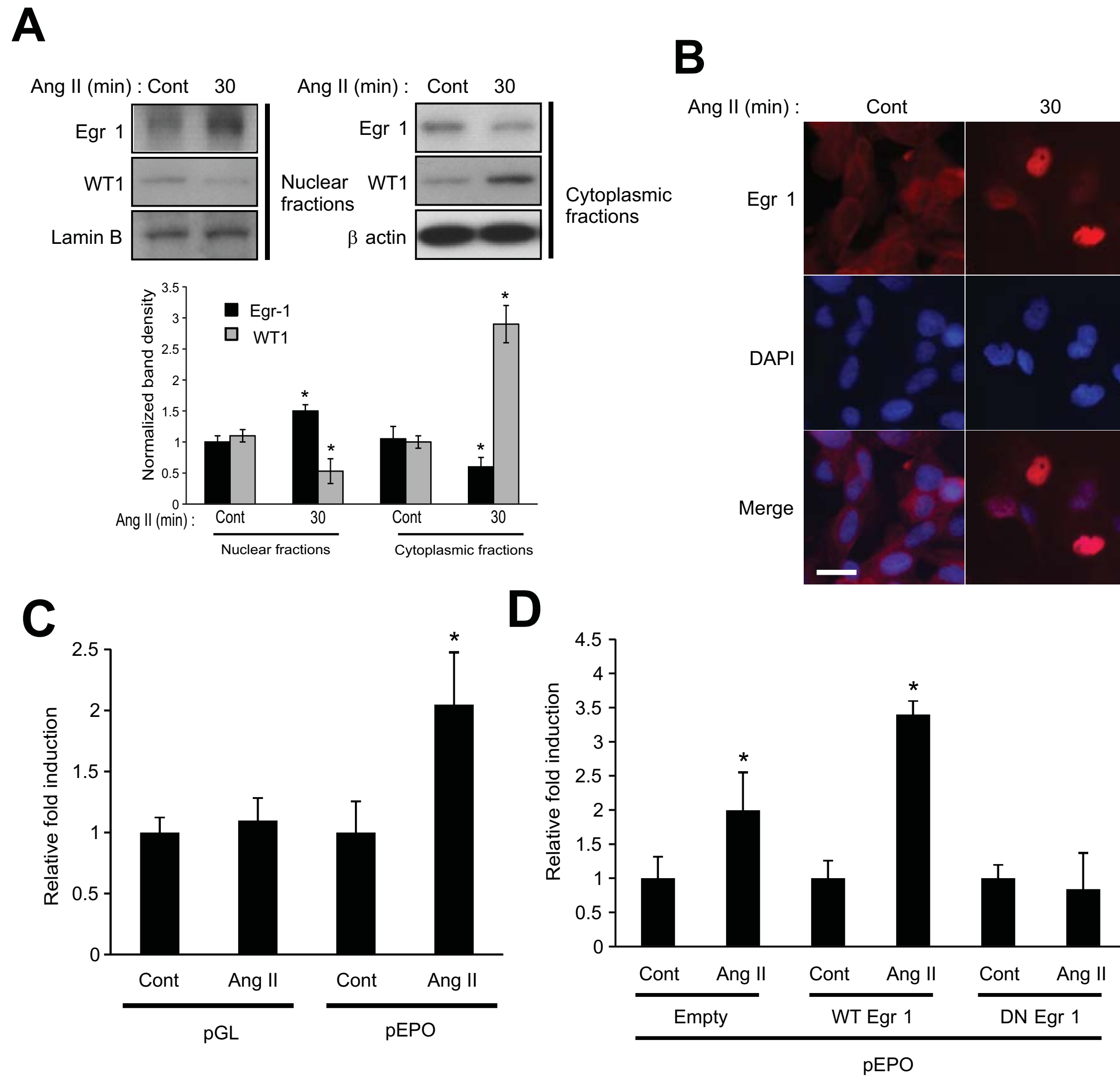

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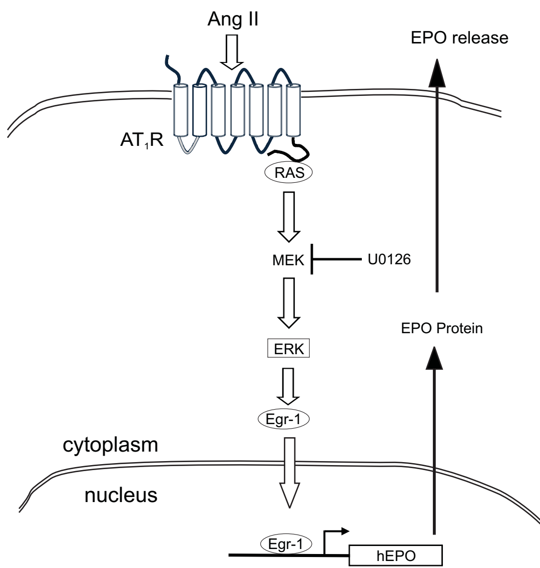
Fig 7



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