Mechanism of Erythropoietin Regulation by Angiotensin II

Yong-Chul Kim, Ognoon Mungunsukh, Elizabeth A. McCart, Peter J. Roehrich, Daniel K. Yee, and Regina M. Day

Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, Maryland (Y.-C.K., O.M., E.A.M., P.J.R., R.M.D.); and Department of Animal Biology, University of Pennsylvania, Philadelphia, Pennsylvania (D.K.Y.)

Received December 5, 2013; accepted April 2, 2014

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 reverse transcription polymerase chain reaction. We further investigated the signaling mechanism of Ang II regulation of EPO in 786-O cells. Pharmacological inhibitors of Ang II type 1 receptor (AT1R) and extracellular signal-regulated kinase 1/2 (ERK1/2) suppressed Ang II transcriptional activation of EPO. Inhibitors of AT1R or Src homology 2 domain–containing tyrosine phosphatase had no effect. Coimmunoprecipitation experiments demonstrated that p21Ras was constitutively bound to the AT1R; this association was increased by Ang II but was reduced by the AT1R inhibitor telmisartan. Transmembrane domain (TM) 2 of AT1R 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 AT1R-mediated Egr-1 activation by p21Ras-mitogen-activated protein kinase/ERK kinase-ERK1/2. Our current data suggest that Ang II, in addition to regulating blood volume and pressure, may be a master regulator of erythropoiesis.

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 and colony-forming unit–erythroid cells, via the EPO receptor (Wu et al., 1995; Jelkmann, 2007). In mice, EPO or EPO receptor 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 a source of EPO in the fetus and prenatal stages of development (Eckardt and Kurtz, 2005). Recombinant human EPO is a clinically significant drug used 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 recombinant human EPO for patient treatment was reported to be ~$6 billion USD/yr between 2006 and 2010 (Lee et al., 2012).

Physiologic 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 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 (Rosenberger et al., 2002; Warnecke et al., 2004; Bunn, 2013). In contrast, GATA transcription factors (GATA-1, -2, and -3) and nuclear factor-κB negatively regulate EPO gene transcription (Imagawa et al., 1997; La Ferla et al., 2002; Obara et al., 2008). Activation of GATA-2 and nuclear factor-κB in response to inflammatory cytokines interleukin-1β and tumor necrosis factor-α

This work was supported by The Defense Medical Research and Development Program [Grant D10 I ARJ7 119] to R.M.D.

The opinions or assertions contained in this paper are the private views of the authors and are not to be construed as reflecting the views, policy, or positions of the Department of the Navy, Department of Defense, nor the U.S. Government. The authors of this manuscript have no conflicts of interest to disclose.
dx.doi.org/10.1124/mol.113.091157.

ABBREVIATIONS: Ang II, angiotensin II; AT-R, angiotensin II type 1 receptor; AT1R, angiotensin II type 2 receptor; EPO, erythropoietin; ERK, extracellular signal-regulated kinase; GPCR, G protein–coupled receptor; GFP, green fluorescent protein; HA, hemagglutinin; HIF, hypoxia-inducible factor; MEK, mitogen-activated protein kinase/ERK kinase; NSC-87877, 8-hydroxy-7-[(6-sulfo-2-naphthyl)azo]-5-quinolinesulfonic acid; PD 123319, 1-[4-(dimethylamino)-3-methylphenyl[methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid difluorooacetate; PKB, protein kinase B; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SHP1/2, c-Src homology-containing protein phosphatase; TM, transmembrane domain; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; WT1, Wilms’ tumor 1.
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 (Ebert and Bunn, 1999; Hardee et al., 2006; Bunn, 2013). 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 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 (Rodgers et al., 2000; Haznedaroğlu and Ozturk, 2003; Beyazit et al., 2007). 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$_1$R) (Freudenthaler et al., 1999, 2000; Gossmann et al., 2001). Furthermore, the Ang II–converting enzyme inhibitors captopril and enalapril that block the maturation of Ang II (Freudenthaler et al., 1999, 2000; Gossmann et al., 2001). EPO protein in situ and in vitro. We further demonstrate that Ang II induces EPO mRNA expression and increases 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 Ang II–converting enzyme 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 in vivo (Kato et al., 2005). Furthermore, EPO synthesis was also found to be reduced in Tsukuba hypertensive mice/AT$_1$R-knockout mice (Kato et al., 2005). We hypothesized that Ang II receptor–dependent signaling is responsible for direct regulation of EPO under nonhypoxic 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$_1$R is required for Egil-1 activation via p21Ras-mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (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 nonhypoxic conditions.

Materials and Methods

Cell Lines and Cell Culture. Human renal 786-O cells were cultured in Dulbecco's modified Eagle's 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 streptomycin (100 μg/ml) in a humidified 5% CO$_2$ incubator at 37°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 (1-[[4-(dimethylamino)-3]-methylphenyl]methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate) were purchased from Sigma-Aldrich (St. Louis, MO). c-Src homology-containing protein phosphatase (SHIP)I/2 PTPase inhibitor, NSC-75777 (3-hydroxy-7-[6-sulfo-2-naphthylamino]-5-quinolinesulfonic acid) was purchased from Calbiochem (San Diego, CA). U0126 (1,4-diamine-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) was from Cell Signaling Technology (Danvers, MA). Antibodies against phospho-Thr202 Tyr204 ERK1/2 (#9101) and phospho-Ser727/221 MEK1/2 (#9154) were from Cell Signaling Technology. Antibodies against ERK1 (sc-94), AT$_1$R (sc-1175, sc-579 and sc-31181 for coimmunoprecipitation), green fluorescent protein (GFP; sc-9996), hemagglutinin (HA; sc-805 for immunoblotting and sc-805-G for coimmunoprecipitation), EPO (sc-7956), Egr-1 (sc-47778), Lamin B (sc-6217), α-tubulin (sc-8035), Gab1 (sc-9049 for Western blots), and Gab1 (sc-6292 for coimmunoprecipitation) were purchased from Santa Cruz Biotechnology, Inc. (San Cruz, CA). Mouse anti-MEK1 and p21Ras antibody was from BD Biosciences (San Jose, CA). We used mouse monoclonal anti–Wilm’s tumor 1 (WT1) antibody (clone 6F-H2) from Millipore (Billerica, MA) and mouse monoclonal anti-HA antibody (12CA5) from Roche Applied Science (Indianapolis, IN).

Plasmids and Transient Transfection. We generated HA-tagged AT$_1$R expression plasmid at XhoI and NotI with phCMV2. Two truncated forms of AT$_1$R (HA-tG306 and tL314; deletion mutant from 306th glycine and 314th leucine of AT$_1$R, respectively) were generated for tG306, forward-5’-GATCTCGAGATCTTCTACTGTTCTTTCTCTACTTGAAGATGTTAT-3’, reverse-5’-GTCGGGCGGCGTCTACCCCAAGAACCATAAAAAAGAAGGA-3’. The tL314, forward-5’-GATCTCAGAGGTCTCAACCTTCTCTTTACTGAGATGTAT-3’, reverse-5’-GTCGAGCGGGCTTGAAGAAAAATCTTTTATTTTTTTTCC-3’. The second transmembrane domain mutant (D74E) and the seventh transmembrane domain mutants (T287V, F293L, and N295V) of AT$_1$R 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 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 transfected using Fugene 6 according to the manufacturer’s recommendation (Roche Applied Science). After 48-hour 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). Kidneys from these 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). Peripheral sections containing the cortex were cut into 1- to 2-mm slices using a McIlwain Tissue chopper (Genex Inc., Montreal, QC, Canada) under a Laminar flow hood. Slices were pooled and randomly divided into 2 x 12-well tissue culture plates (3–6 slices per well) containing Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal bovine serum. Slices were incubated for 30 minutes at 37°C in a humidified cell culture incubator prior to treatments. Ang II treatments were performed in the presence of 10% serum.

Quantitative Reverse-Transcription Polymerase Chain Reaction. 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 spectrophotometrically (ND-1000 Spectrophotometer; NanoDrop,
plasmids were cotransfected (pRL-TK, 150 ng/well). Luciferase and To control for transfection efficiency, a 24-well plate using the Fugene 6 reagent (Roche Applied Science). Additionally, control for transfection efficiency, a 24-well plate using the Fugene 6 reagent (Roche Applied Science). Primers for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) were designed using the ProbeFinder software, version 2.25 (Roche Applied Sciences). Primer pairs for murine EPO gene expression were forward-5'-TCTTGAGAAGAGCGAGTCTCG-3' and reverse-5'-TCTTCTGCAACACCATCTG-3'; human EPO gene expression: hEPO, forward-5'-CGTGCTAGCACGCA-3' and reverse-5'-CCCTGGCAGACCTTCACGG-3'. As internal control, mRNA levels of g-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.

**Commuunoprecipitation and Immunoblotting Assay.** 786-O cells were grown to 80% confluence prior to treatment. Where noted, cells were pretreated for 1 hour with NSC-87877 or U0126 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. Coimmunoprecipitation and immunoblotting were performed as described previously (Kim et al., 2011; Kim and Day, 2012).

**Luciferase Assay.** The luciferase-linked human EPO promoter plasmid pEPO (−292 to −7 bp) was constructed in pGL3-basic vector (Promega, Madison, WI). The oligonucleotides primers used for cloning the EPO promoter were forward-5'-CGTGCTAGCACGCAACATGGACATAACAG-3' and reverse-5'-GGAAAGCTTGCTGGGCCGGGTTCCCTCAGC-3'; the promoter was cloned into the NheI-HindIII sites of pGL3. The construct was sequenced using a Big Dye sequencing kit (Invitrogen). To analyze reporter activation, 0.2 μg/well of the pEPO reporter plasmid was transfected into 786-O cells in a 24-well plate using the Fugene 6 reagent (Roche Applied Science). To control for transfection efficiency, Renilla luciferase gene expression plasmids were cotransfected (pRL-TK, 150 ng/well). Luciferase and Renilla activities were measured using a dual luciferase system (Promega).

**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). 4',6-Diamidino-2-phenylindole 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 postanalysis as appropriate. P values were calculated by means ± S.D., 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 cultured for 30 minutes prior to treatment with Ang II for 4 hours. Total RNA was isolated, and EPO mRNA levels were measured using qRT-PCR (Fig. 1A). CoCl2 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 hours. A time course revealed that Ang II treatment increased EPO mRNA expression within 2 hours, maximal for 4 hours (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 hour, suggesting that a nontranscriptional mechanism for EPO regulation may also be involved in whole tissue. We next investigated Ang II regulation of EPO in 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 hours, and 50 μM Ang II activated expression of EPO gene by ∼4.5-fold within 6 hours (Fig. 1D). EPO protein was increased ∼1.6-fold within 12 hours (Fig. 1E).

**Ang II-Induced Activation of EPO Requires AT1R and the MEK1/2/ERK1/2 Signaling Pathway.** The biologic actions of Ang II are mediated primarily by two specific receptors, angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors (Mogi et al., 2007). Both AT1R and AT2R belong to the seven transmembrane G protein–coupled receptor (GPCR) family, and the activation of AT1R or AT2R downstream signaling by Ang II has been demonstrated to be cell type and tissue type specific (Mogi et al., 2007; Lee et al., 2010; Kim and Day, 2012). 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 AT2R inhibitor telmisartan. In contrast, Ang II–induced EPO levels were further increased ~1.6-fold by the AT2R specific blocker PD 123319, compared with Ang II alone (Fig. 2A). AT1R and AT2R 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.

AT1R has been demonstrated to signal through a variety of downstream molecules, including GTP-binding proteins, 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. 2B). Our laboratory previously demonstrated that SHP1/2 activation is important for Ang II signaling in 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 AT1R 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 AT1R in Ang II regulation of platelet-derived growth factor and plasminogen activator inhibitor-1 gene expression (Day et al., 1999; Chen and Feener, 2004). 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 minutes after Ang II treatment and was blocked by the presence of telmisartan (Fig. 2D). Phospho-ERK1/2 (Thr202/Tyr204) was induced within 15 minutes 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/Akt), AMP-dependent protein kinase, p38 MAPK, and c-Jun N-terminal kinase/stress-activated protein kinase (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.
Ang II induces both mRNA and protein levels of EPO in vivo and in vitro. (A and B) Ang II–induced transcriptional EPO gene expression in murine kidney slices. Kidney slices were incubated in culture medium for 30 minutes 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 (CoCl₂; 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 ± S.D. *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 hours. Bar graphs show the fold change of EPO expression and was quantifying as the ratio to the control (Cont). The data show means ± S.D. *P < 0.05 versus the respective control, n = 3. (C) Protein levels of EPO were determined at the indicated time points after 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 ± S.D., n = 3. *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 or 50 µM of Ang II. CoCl₂ (60 µM) was used as positive control to induce EPO. Total mRNA was isolated after 2, 4, 6, 12, or 24 hours. 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 means ± S.D., n = 3. *P < 0.05 versus control. (E) Protein levels of EPO were determined at the indicated time points after 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 ± S.D., n = 3. *P < 0.05 versus control.
p21Ras Is a Primary Upstream Regulator of Ang II–Induced Activation of ERK1/2, and Ang II Regulates AT1R-p21Ras Interaction. Both heterotrimeric G proteins and the small monomeric G protein Ras have been demonstrated to be activated by GPCRs (Marty and Ye, 2010; Dong and Wu, 2013). We investigated the potential role of p21Ras activation by AT1R 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 hours 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 AT1R and p21Ras for MAPK activation in neurons (Yang et al., 1996). We investigated the association of endogenous AT1R with p21Ras in 786-O cells. The interaction between AT1R and p21Ras was confirmed by immunoprecipitation using an antibody against the C terminus of AT1R (Fig. 3B). Interestingly, the association between p21Ras and AT1R was increased by Ang II within 5 minutes and was completely inhibited by telmisartan (Fig. 3B). The interaction was confirmed using exogenous expression of GFP-tagged p21Ras.
**Fig. 3.** Ang II regulates interactions between AT1R 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 hours. Cells were treated with Ang II (50 μM) for 15 minutes. Whole-cell lysates were Western blotted for phosphorylated ERK1/2 (p-ERK1/2-Thr202/Tyr204) or EPO. Membranes were reblotted for ERK1, β-actin, or GFP as internal controls.

(B) IP: AT1R
- Input:
  - IgG
  - Telmisartan
  - Ang II

IB: p21Ras
- N-terminus of AT1R
- C-terminus of AT1R (360-359)

(C) IP: GFP
- Input:
  - Telmisartan
  - Ang II

IB: HA
- AT1R

IB: GFP
- p21Ras

Heavy chain IgG

(D) WT-AT1R: HA
- M.W.: 55

306 359

TG306: HA
- M.W.: 55

306 359

TL314: HA
- M.W.: 55

36 55

Heavy chain IgG

**Legend:** M.W. indicates molecular weight.
and HA-tagged AT1R. Treatment of transfected cells with Ang II increased the association between HA-AT1R and GFP-p21Ras, but treatment with telmisartan reduced this interaction (Fig. 3C), consistent with our findings with endogenous Ras antibodies. We examined the binding of GFP-p21Ras with HA-tagged wild-type AT1R or two mutants of AT1R truncated in the cytoplasmic C-terminal domain [one truncated after G306 (G306) and the other truncated after L314 (L314)]. 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 (Seta et al., 2002; Becker et al., 2004). Coimmunoprecipitation of p21Ras was reduced (>60%) by both truncation mutants (Fig. 3D). This suggests that the C terminus of AT1R 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 AT1R and regulation by Ang II. Reciprocal coimmunoprecipitation with anti-AT1R (C-terminal cytoplasmic 306–359 regions) or Gab1 antibodies for endogenous AT1R and Gab1 interaction indicated that Gab1 was constitutively associated with AT1R in 786-O cells (Fig. 4). Unlike the AT1R-p21Ras interaction, Ang II and telmisartan had no effect on Gab1 association (data not shown).

**The D74 Mutant in the Transmembrane Domain Two of AT1R 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 AT1R 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 AT1R for ERK activation and/or for G protein/phospholipase C/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 AT1R, and this amino acid is highly conserved in among G protein–coupled receptors (Bihoreau et al., 1993; Aplin et al., 2009). We used ectopic expression of a number of AT1R mutants to identify regions of the receptor required for ERK1/2 activation in 786-O cells. The cells were transfected with wild-type AT1R (AT1R) or a mutant of TM2 (D74E) or mutants of TM7 (T287V, F293L, or N295S) for 48 hours (Yee et al., 2006). ERK activation was determined after Ang II stimulation for 15 minutes, 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 AT1R (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.** Egr-1 transcription factor mediates Ang II regulation of platelet-derived growth factor A-chain and c-kit D in vascular smooth muscle cells and in Chinese hamster ovary cells, 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 minutes of Ang II stimulation (Fig. 6A). Since the WT1 transcriptional activation 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 to −7 bp). Ang II increased the expression of pEPO ~2-fold (Fig. 6C). Overexpression of wild-type 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 cotransfection of dominant-negative 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.

**Discussion**

Erythropoietin has been extensively studied for its role in erythropoiesis, and increasing evidence has demonstrated that EPO also has critical biologic functions in other cell types, including neurons, endothelial cells, and epithelial cells from a variety of tissues (Sirén et al., 2001; Brines and Cerami, 2006; Wang et al., 2009). Despite the expanding insights into the role of EPO in overall biologic homeostasis, the mechanism of basal (nonhypoxic) regulation of EPO is not understood. Clinical and preclinical studies have provided evidence that EPO is regulated by Ang II levels in the plasma.
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 AT1R-mediated 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 (Rodgers et al., 2000; Haznedaroglu and Ozturk, 2003; Beyazit et al., 2007). 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 (Neves et al., 2002; Waters et al., 2004; Dong and Wu, 2013). GPCRs have been shown to broadly regulate heterotrimeric G proteins in the physiologic 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 (Esseltine et al., 2011; Dong and Wu, 2013).

The structural determinants for AT1R activation of the ERK1/2 signaling pathway remain controversial (Belcheva and Coscia, 2002; Yee et al., 2006). AT1R 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 AT1R in different cellular backgrounds (Doan et al., 2001). p21Ras association with AT1R was increased in the presence of Ang II but blocked in the

![Image](https://example.com/fig4.png)

**Fig. 4.** AT1R associates with Gab1. Endogenous AT1R was immunoprecipitated (IP) followed by immunoblotting (IB) for Gab1 or for AT1R as a loading control (left). Endogenous Gab1 was immunoprecipitated followed by immunoblotting for AT1R or for Gab1 as a loading control (right).

![Image](https://example.com/fig5.png)

**Fig. 5.** D74E mutant of the TM2 of AT1R is involved in ERK activation by Ang II. 786-O cells were grown to 70% confluence before transfected with wild-type AT1R (AT1R) (A) or empty vector, AT1R, T287V, D74E, F293L, or N295S (B) for 48 hours, subsequently treated with Ang II (50 μM) for an additional 15 minutes 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 reblot 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 ± S.D. *P < 0.05 versus the basal control of cells transfected with wild-type AT1R; †P < 0.05 for Ang II treatment versus untreated control in the same transfection, n = 3.
presence of the AT1R 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 AT1R 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 AT1R 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

Fig. 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 minutes and nuclear and cytoplasmic fractions were prepared for Western blotting for Egr-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 ± S.D. *P < 0.05 versus the respective control, n = 3. (B) 786-O cells were treated with 50 μM Ang II for 30 minutes 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, BC, Canada) with iVision-Mac 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 hours, cells were treated with or without Ang II (50 μM) for 24 hours. Bar graphs indicate the relative induction of luciferase normalized to Renilla. Graphs show means ± S.D. *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 hours, cells were treated with or without Ang II (50 μM) for 24 hours. Bar graphs show the relative induction of EPO promoter-driven luciferase activity normalized to Renilla activity. Graphs show means ± S.D. *P < 0.05 for Ang II treatment versus the respective transfection control, n = 3.
AT1R may be involved in p21Ras binding but does not and tL314. This finding suggests that the C-terminal region of p21Ras interaction was reduced by truncation mutants tG306.

Ang II is more complex, including multiple mechanisms of the active AT1R conformation, whereas the interaction between AT1R and Gab1 is constitutive and does not modulate by Ang II or telmisartan stimulation (data not shown). Overall, we interpret our data to indicate that AT1R and Gab1 directly interacts with the Gab1 docking protein. This interaction is constitutive and not modulated by Ang II or telmisartan stimulation (data not shown).

Fig. 7. Schematic model of Ang II–induced regulation of EPO in 786-O cells. Proposed mechanism for EPO regulation upon Ang II stimulation via physiologic interaction between AT1R and p21Ras. Ang II binding to AT1R 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.

a G protein–dependent pathway is the primary mechanism by which Ang II activates ERK1/2 in 786-O cells through AT1R.

Our structural studies also demonstrated that the AT1R-p21Ras interaction was reduced by truncation mutants tG306 and tL314. This finding suggests that the C-terminal region of AT1R may be involved in p21Ras binding but does not completely encompass the binding elements. Thomas et al. (1995) showed that truncation of the last 45 amino acids of AT1R (tL314) affected Ang II–induced AT1R endocytosis but had little effect on the G protein coupling in Chinese hamster ovary-K1 cells (Thomas et al., 1995). Another study indicated that the third intracellular cytoplasmic loop domain of AT1R 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 AT1R directly interacts with the Gab1 docking protein. This interaction is constitutive and not modulated by Ang II or telmisartan stimulation (data not shown). Overall, we interpret our data to indicate that AT1R and Gab1 are directly and constitutive and does not require Ang II–induced AT1R conformational activation.

Our results in 786-O cells suggest that p21Ras-ERK1/2-Egr-1 signaling increases EPO expression through gene transcription. Interestingly, in kidney slice cultures, Ang II increased EPO protein levels within 1 hour, prior to the observed increase in EPO mRNA, suggesting that additional post-transcriptional 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.