Angiotensin II Stimulates Hypoxia-Inducible Factor 1α Accumulation in Glomerular Mesangial Cells

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ABSTRACT: **Hypoxia increases hypoxia-inducible factor 1α (HIF-1α) protein levels by inhibiting ubiquitination and degradation of HIF-1α, which regulates the transcription of many genes. Recent studies have revealed that many ligands can stimulate HIF-1α accumulation under nonhypoxic conditions. In this study, we show that angiotensin II (Ang II) increased HIF-1α protein levels in a time- and dose-dependent manner under normoxic conditions. Treatment of mesangial cells with Ang II (100 nM) increased production of reactive oxygen species (ROS). Ang II (100 nM) increased the phosphorylation of PDK-1 and Akt/PKB in glomerular mesangial cells. Ang II–stimulated HIF-1α accumulation was blocked by the phosphatidylinositol 3-kinase (PI-3K) inhibitors, Ly 294001, and wortmannin, suggesting that PI-3K was involved. Because increased ROS generation by Ang II may activate the PI-3K–PKB/Akt signaling pathway, these results suggest that Ang II may stimulate a ROS-dependent activation of the PI-3K–PKB/Akt pathway, which leads to HIF-1α accumulation.**

KEYWORDS: **angiotensin II; hypoxia-inducible factor 1; phosphatidylinositol 3-kinase; Akt; reactive oxygen species**

INTRODUCTION

Angiotensin II (Ang II) plays a central role in the pathophysiology of renal diseases. In addition to its hemodynamic actions, Ang II exerts several nonhemodynamic effects. Ang II causes mesangial cell proliferation and regulates the expression of

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several genes that are involved in intracellular signaling cascades in glomerular mesangial cells.[1] Recently, it has been shown that Ang II is involved in the process of tissue destruction in chronic renal diseases[2] and that angiotensin-converting enzyme inhibitors slow the progress of renal diseases.[3] One of the Ang II–induced pathological effects is mediated through expression of vascular endothelial growth factor (VEGF). Ang II–induced VEGF expression has been attributed to the pathogenesis of diabetic nephropathies.[4] VEGF is involved in angiogenesis, wound healing, and inflammation, and it plays a major role in a chronic cyclosporine nephrotoxicity and diabetes-associated microvasculopathies and glomerulosclerosis.[5,6]

VEGF is transcriptionally regulated by hypoxia-inducible factor 1 (HIF-1).[6] HIF-1 is a heterodimer composed of the basic helix–loop–helix proteins HIF-1α and the aryl hydrocarbon nuclear translocator that is also known as HIF-1β.[7] An active HIF-1 heterodimer binds to the HIF-1 binding site within the hypoxia response element and enhances transcription of hypoxia-inducible genes involved in glucose/energy metabolism, cell proliferation and viability, erythropoiesis, iron metabolism, vascular development, or remodeling. Whereas HIF-1β is found in most cells, HIF-1α is undetectable in normoxic conditions. The availability of HIF-1α is determined predominantly by stability regulation of HIF-1α via proline hydroxylation.[8] HIF-1α is degraded under normoxic conditions by von Hippel–Lindau protein (pVHL). In addition to hypoxia, mitochondrial generation of reactive oxygen species (ROS), including superoxide and H_2O_2, may also cause HIF-1 accumulation and subsequent expression of genes inducible by HIF-1 activation under normoxic conditions.[9]

Several signaling pathways, including phosphatidylinositol 3-kinase (PI-3K), serine/threonine kinases (e.g., protein kinase C), and mitogen-activated protein kinase (MAPK) have been implicated as the signal transduction pathways in the regulation of HIF-1α accumulation in a cell-specific manner.[10–12] In this study, we demonstrated that Ang II stimulated ROS production and activated the PI-3K–PDK-1–PKB/Akt pathway, leading to increased HIF-1α accumulation in glomerular mesangial cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), and other reagents used in cell cultures were purchased from Life Technologies (Gaithersburg, MD); antibodies specific for α-tubulin, p38 MAPK, phospho-PDK-1, and phospho-Akt/protein kinase B (PKB) antibodies from Transduction Laboratory (Lexington, KY); Ly 294002, PD 98059, SB 203580, and wortmannin from Calbiochem (San Diego, CA); horseradish peroxidase–conjugated anti–rabbit immunoglobulin G (IgG) antibody from Bio-Rad (Hercules, CA); 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium substrate from Kirkegaard and Perry Laboratories (Gaithersburg, MD); chemiluminescence kits from Amersham (Buckinghamshire, UK); 2′,7′-dichlorofluorescein diacetate (DCFH-DA) from Molecular Probes (Eugene, OR); protease inhibitor cocktail tablets from Boehringer Mannheim (Mannheim, Germany); and Ang II and all other chemicals from Sigma (St. Louis, MO).

Cell Culture and Preparation of Cell Lysates

Simian virus 40–transformed mesangial cells were cultured in Leibovitz's L-15 medium supplemented with 13.1 mM $NaHCO_3$, 13 mM glucose, 2 mM glutamine, 10% heat-inactivated fetal bovine serum, and penicillin (100 U/mL)–streptomycin (100 mg/mL). Cells were plated at a concentration of 10^5 cells/mL and maintained in a humidified incubator under a 5% CO_2 atmosphere at 37°C. Cells were used for experiment when they reached approximately 80% confluence. All reagents were added directly to the culture at a volume of 100 µL/10 mL of medium. For preparation of cell lysates, cells were harvested, chilled on ice, and washed three times with ice-cold phosphate-buffered saline (PBS). Later procedures were carried out on ice unless otherwise specified. Cells were lysed by adding lysis buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.1% mercaptoethanol, 0.5% Triton X-100, and the protease inhibitor cocktails (final concentrations: 0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 50 µg/mL leupeptin). Cell lysates were stored at –70°C for further measurements.

Polyacrylamide Gel Electrophoresis and Western Blotting

Electrophoresis was carried out with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (7.5%). Following electrophoresis, proteins on the gel were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. After transfer, the PVDF paper was washed once with PBS and twice with PBS plus 0.1% Tween 20. The PVDF membrane was then blocked with blocking solution containing 3% bovine serum albumin in PBS containing 0.1% Tween 20 for 1 h at room temperature. The PVDF membrane was incubated with a solution containing primary antibodies in the blocking buffer. Finally, the PVDF paper was incubated with peroxidase-linked anti–mouse IgG antibodies for 1 h and then developed using a commercially available chemiluminescence kit.

Measurement of Intracellular ROS Generation

Intracellular ROS generation was assessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as described by Chandel *et al.*[10] In brief, mesangial cells were cultured in petri dishes and incubated with 10 µM DCFH-DA for 30 min. Cells were washed and incubated with Ang II (100 nM) for various periods of time. ROS in the cells caused oxidation of DCFH, generating a fluorescent product (DCF). The intracellular fluorescence of DCF was then measured using flow cytometry. Data were normalized to fluorescence intensities obtained from untreated control subjects.

RESULTS

Ang II Effect on HIF-1α Protein Levels

In glomerular mesangial cells deprived of FCS for 16 h, the protein level of HIF-1α was barely detected under normoxic conditions. Exposure of serum-deprived mesangial cells to Ang II or $CoCl_2$ rapidly increased HIF-1α cellular protein levels. The

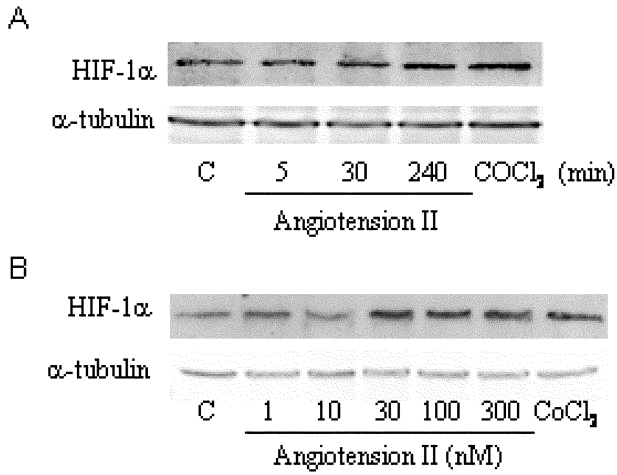

FIGURE 1. HIF-1α protein levels in glomerular mesangial cells. (**A**) Cells were incubated with 100 nM of Ang II for different periods. After incubation, the cells were lysed, and protein levels of HIF-1α were immunodetected with HIF-1α–specific antibody. (**B**) Glomerular mesangial cells were incubated with various concentrations of Ang II for 4 h, and protein levels of HIF-1α were immunodetected with HIF-1α–specific antibody using Western blot analysis. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

maximum response was seen 4 h after Ang II exposure (FIG. 1A). Ang II (30–300 nM) increased HIF-1α cellular protein levels in a dose-dependent manner. The maximal effect was seen at a concentration of 100 nM (FIG. 1B).

Ang II Effect on Intracellular ROS Generation

Ang II has been shown to modulate the NAD(P)H oxidase system and increase intracellular ROS levels in several cell types. We next investigated whether Ang II increased the intracellular ROS levels using DCFH-DA. As shown in FIGURE 2, treatment of mesangial cells with Ang II increased the intracellular ROS production. The increase in DCF fluorescence induced by Ang II became evident at 30 min and reached the maximum at 4 h.

Ang II Activation of PI-3K Pathway

We then examined whether Ang II stimulates the PI-3K pathway. Activation of PI-3K may activate and phosphorylate PDK-1, which in turn phosphorylates the downstream PKB/Akt kinase at specific phosphorylation sites on Ser^{473}. As depicted in FIGURE 3, incubation of mesangial cells with Ang II increased PDK-1 and PKB/Akt phosphorylation in a dose-dependent manner. Ang II did not increase the protein level of PI-3K.

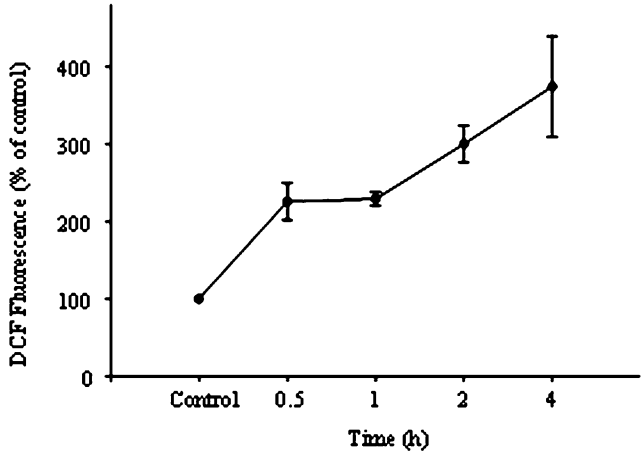

FIGURE 2. Ang II increases intracellular ROS generation in glomerular mesangial cells. Cells were incubated with DCFH-DA (10 μM) for 6 h in the presence of Ang II (100 nM) for various periods. Results are expressed relative to untreated control and are expressed as the mean ± SEM of three independent experiments performed in triplicate. $*P < 0.05$ compared with untreated control.

PI-3K–dependent Stimulation of HIF-1α Accumulation by Ang II

To investigate the link between Ang II–stimulated PI-3K activation and increases in HIF-1α protein levels, we pretreated mesangial cells with specific pharmacological inhibitors of PI-3K, Ly 294002, and wortmannin. As shown in FIGURE 4, Ang II–stimulated PKB/Akt phosphorylation was inhibited with Ly 294002 or wortmannin pretreatment. In accordance with this observation, Ang II–stimulated HIF-1α accumulation was also blocked by Ly 294002 (50 μM), suggesting that PI-3K plays an important role in mediating HIF-1α accumulation.

DISCUSSION

Ang II plays a key role in the regulation of fluid and electrolyte balance and has been implicated in the pathogenesis of renal diseases. HIF-1 controls the expression of several genes under hypoxic conditions and has been linked to Ang II activity in renal diseases. However, the roles of HIF-1 and the cellular signaling mechanisms that regulate the increase of HIF-1α protein level by Ang II in mesangial cells have not been elucidated. In this study, we demonstrate that Ang II increased HIF-1α protein levels in cultured mesangial cells under normoxic conditions. We present data showing that an increase in the HIF-1α protein level was accompanied by an increase in ROS generation and the activation of the PI-3K pathway. Thus, Ang II may bypass hypoxic condition to increase HIF-1α protein levels, which in turn lead to induction of HIF-1α–responsive genes such as VEGF, resulting in several pathological conditions.

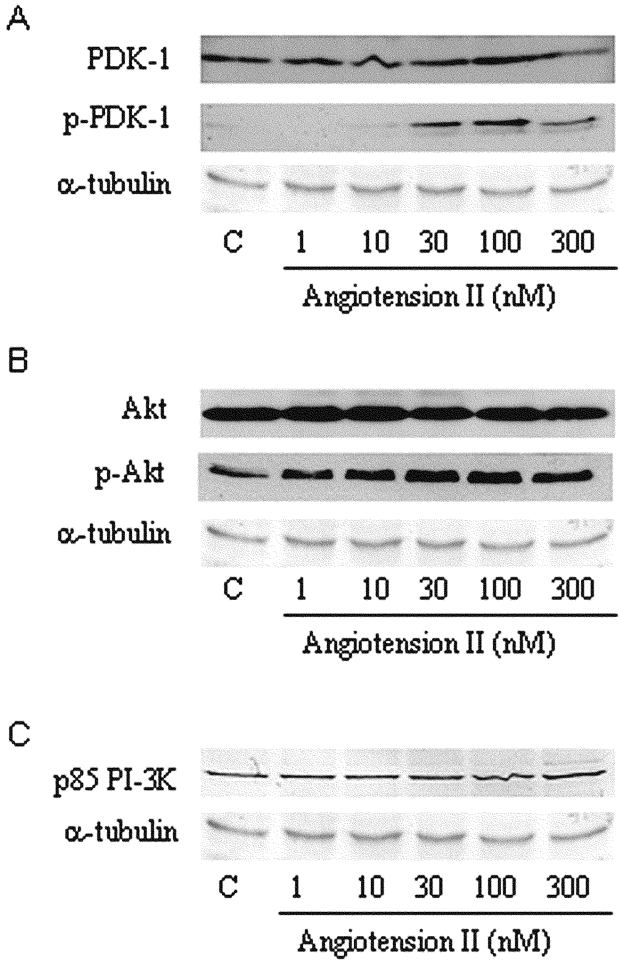

FIGURE 3. Ang II stimulates PI-3K signaling pathway in mesangial cells. (**A**) Cells were incubated with various concentrations of Ang II for 30 min. After incubation, the cells were lysed and then immunodetected with PDK1 and p-PDK1–specific antibody (**A**) or with Akt and p-Akt473–specific antibody (**B**), or p85 PI-3K–specific antibody (**C**). Equal loading in each lane was demonstrated by the similar intensities of α–tubulin.

Free radicals have been shown to play a crucial role in mediating several signaling pathways. We demonstrated that Ang II increased the ROS production in glomerular mesangial cells. In vascular smooth muscle cells, Ang II increases the HIF-1α protein level by a ROS-dependent activation of the PI-3K pathway.[11] In glomerular mesangial cells, increased generation of ROS by Ang II may activate the PI-3K pathway as well. Activation of the PI-3K pathway may modulate the activation of eukaryotic translation initiation factor 4F (eIF-4F) and/or the ribosomal S6 protein by

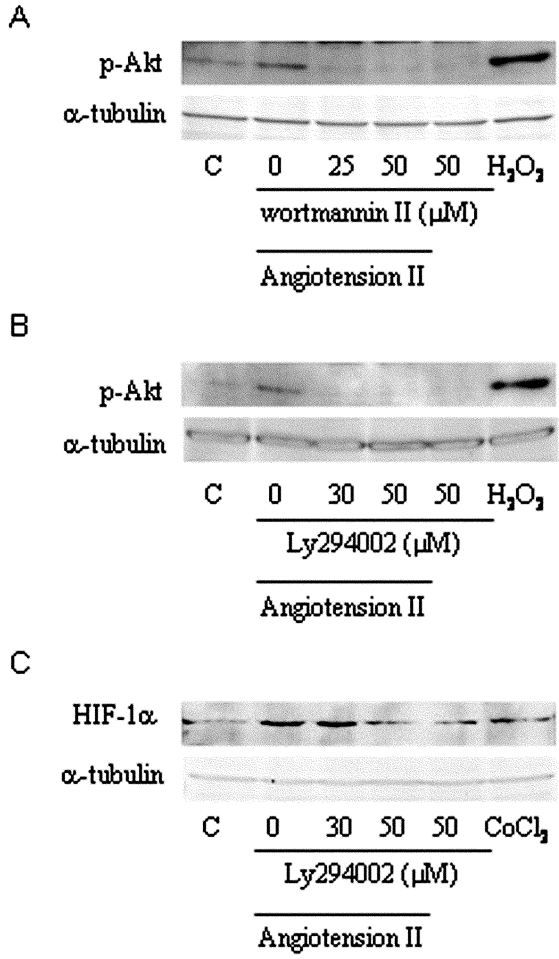

FIGURE 4. Effects of wortmannin on Ang II–stimulated increase in p-Akt473 activity and HIF-1α accumulation in rat mesangial cells. In (**A**) and (**B**), mesangial cells were pretreated with wortmannin (0–50 μM) or Ly 294002 (0–50 μM) for 30 min before the addition of 100 nM Ang II for 30 min. After incubation, the cells were lysed, and the p-Akt473 activity was determined with an immunodetected p-Akt473–specific antibody as described in MATERIALS AND METHODS. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin. In (**C**), mesangial cells were pretreated with Ly 294002 (0–50 μM) for 30 min before the addition of 100 nM Ang II for 4 h. After incubation, the cells were lysed, and the HIF-1α expression was determined with an HIF-1α–specific antibody as described in MATERIALS AND METHODS. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

the PI-3K/p70S6K/mTOR pathway and subsequently increase HIF-1α protein level.[11]

Ang II activates many signaling pathways. PI-3K/Akt is a common signaling pathway activated by a variety of ligands and is involved in transcriptional regulation of VEGF in the kidney.[11] We demonstrate that incubation of mesangial cells with specific inhibitors of PI-3K potently blocked the increase in HIF-1α protein levels by Ang II, suggesting that PI-3K is involved in Ang II–induced HIF-1α accumulation. We also show that treatment of mesangial cells with Ang II stimulated the phosphorylation of PDK-1 and PKB/Akt, both downstream effectors in the PI-3K pathway. These data agree with those found in vascular smooth muscle cells showing that the PI-3K pathway can be activated by Ang II.[11,12]

In conclusion, our data suggest that Ang II increases ROS production and activates the PI-3K signaling pathway, leading to an increase in HIF-1α accumulation. Given the role of HIF-1α in controlling the expression of several genes that are involved in the pathophysiology of renal diseases, Ang II may exert its pathogenetic role via the regulation of HIF-1α accumulation.

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