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Angiotensin II Regulates Vascular Endothelial Growth Factor via Hypoxia-Inducible Factor-1α Induction and Redox Mechanisms in the Kidney

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ABSTRACT

Angiotensin II (AngII) is a cytokine that participates in renal damage. Vascular endothelial growth factor (VEGF) is constitutively expressed in the kidney and is involved in the progression of renal disease. The aim of this work was to investigate the relation between AngII and VEGF and the mechanisms involved in its regulation in the kidney. We have observed that in cultured tubuloepithelial cells (NRK52E cell line) AngII increased VEGF gene expression and promoter activation. Hypoxia-inducible factor-1 (HIF-1) is one of the main VEGF gene activators. AngII induces HIF-1α protein production and increases HIF-1 DNA-binding activity. An antisense HIF-1α oligodeoxynucleotide inhibited AngII-induced VEGF overexpression. The reactive oxygen species act as second messengers in kidney damage caused by AngII and mediate the induction of HIF-1 by cytokines. In tubuloepithelial cells, VEGF up-regulation and HIF-1α induction due to AngII were significantly diminished by antioxidants, suggesting a redox-mediated mechanism. Infusion of AngII into mice caused renal VEGF overexpression, HIF-1 activation, and oxidative stress. In summary, these data show that AngII *in vivo* and *in vitro* up-regulates renal VEGF expression by a mechanism that involves HIF-1 activation and oxidative stress. *Antioxid. Redox Signal.* 7, 1275–1284.

INTRODUCTION

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) is an endothelial specific growth factor that regulates proliferation, migration, permeability, differentiation, survival, and interstitial matrix remodeling in endothelial cells (EC) (2, 5, 11, 40). VEGF binds to a family of tyrosine kinases receptors (VEGFR1, VEGFR2, VEGFR3, and a complementary receptor neurophilin-1). Each of these receptors has different signal transduction properties and functions; VEGFR2 is the most important in functional terms (2, 11, 25, 40). In the kidney, VEGF expression is detected predominantly in glomerular podocytes and tubular epithelial cells, whereas VEGF receptors are found in glomerular and peritubular EC (33). The role of VEGF in normal renal physiology is not clearly defined (16, 33). In renal pathophysiology, the integrity of the microvasculature is not correlated with the stereotyped answer of renal VEGF expression after renal damage (16, 33). VEGF inhibition has beneficial effects on diabetic-induced nephropathy (8). However, VEGF is required for glomerular and tubular hypertrophy and proliferation in response to nephron reduction (12, 17, 23) and glomerular and tubulointerstitial repair in cyclosporine nephrotoxicity (1, 17, 23). At the same time, VEGF could be a mediator of profibrotic and inflammatory factors, including angiotensin II (AngII) (16, 42).

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AngII plays an important role in kidney damage progression through the regulation of cell growth, fibrosis, and inflammation (21). In diverse tissues, such as vascular smooth muscle cells (VSMC) and EC, AngII up-regulates VEGF expression and synthesis (4, 19, 29, 38). The relation between AngII and VEGF in the kidney has recently been described (19, 29). The blockade of AngII receptors (AT_1 and AT_2) diminished renal VEGF overexpression in AngII-infused rats (29). However, the molecular mechanisms involved in this process have not been determined.

Hypoxia-inducible factor-1 (HIF-1) is the best characterized regulator of the VEGF gene transcription (20). HIF-1 transcription factor is a heterodimer composed of HIF-1α and HIF-1β. HIF-1β protein is found in all cells, whereas HIF-1α is virtually undetectable in normal oxygen conditions. Under hypoxic conditions, active HIF-1 complexes accumulate in the cell nucleus. They bind to the target DNA sequence (HIF-1 binding site) within the hypoxia-response element and enhance the hypoxia-inducible gene transcription rate (15). Some evidence suggests that nonhypoxic stimuli can also activate HIF-1α in a cell-specific manner (28). In this way, AngII activates HIF-1α in VSMC and regulates VEGF (3, 24). However, there are no studies about renal cells or the kidney.

One of the most important mechanisms involved in AngII-induced renal damage is the production of reactive oxygen species (ROS), which can act as intracellular signaling molecules (31, 36). ROS can activate multiple intracellular proteins, enzymes, and transcription factors, including HIF-1, activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (31), and genes such as VEGF (28). ROS control cell growth, apoptosis, hypertrophy, migration, inflammation, and fibrosis (26). AngII, through induction of ROS, regulates the expression of redox-sensitive inflammatory genes, such as monocyte chemoattractant protein-1 (35) and interleukin-6 (31), and profibrotic genes, such as connective tissue growth factor (32).

In this work, we investigated the mechanisms of AngII-mediated VEGF gene regulation in the kidney *in vivo* and *in vitro*, evaluating the potential role of several transcription factors, especially HIF-1, and the involvement of ROS production.

MATERIALS AND METHODS

Experimental design

In vivo. Studies were performed in adult male C57Bl/6 mice (9–12 weeks old, 20 g; Harlan Interfauna Iberica, S.A., Barcelona, Spain). All the procedures on animals were performed according to the international and institutional Animal Research Committee guidelines.

Systemic infusion of AngII was done into mice by subcutaneous osmotic minipumps (Alza Corp., Cupertino, CA, U.S.A.), at the dose of 1,000 ng/kg/min. Animals were killed at 3 days ($n = 8$ in each group). Control animals of the same age were also studied. To determine the AngII receptors, the AT_1 antagonist losartan (10 mg/kg/day; drinking water) or the AT_2 antagonist PD123319 (30 mg/kg/day, s.c. osmotic minipumps) were used, starting 24 h before AngII infusion, at doses that effectively blocked each receptor (9). Losartan was kindly provided by Merck Sharp & Dohme (Madrid, Spain), and PD123319 was from Sigma (St. Louis, MO, U.S.A.). The doses used for antagonists alone did not cause renal damage (data not shown).

In vitro. Tubuloepithelial cells [normal rat kidney tubular epithelial cell 52E (NRK52E) cell line] were used. Cells were grown in Dulbecco's modified Eagle medium plus 10% fetal bovine serum (FBS) (Innogenetics, Barcelona, Spain). For experiments, cells were used at 80% confluence and growth arrested by serum starvation for 24 h. They were treated with 10^{-7} mol/L AngII or 10^{-4} mol/L $CoCl_2$ as positive control (Sigma). The phosphorothioate-modified oligodeoxynucleotides (ODNs) were synthesized by Pacisa y Giralt (Madrid, Spain). Cells were preincubated for 1 hour with antioxidants (Sigma) and/or 30 min with the AT_1 and AT_2 blockers.

Gene studies

mRNA isolation, real-time reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA was isolated with TRIZOL (Invitrogen, San Diego, CA, U.S.A.). Real-time RT-PCR was performed using a fluorogenic TaqMan MGB probe, and primers (rat VEGF, mouse VEGF, mouse VEGFR2, mouse VEGFR1, and 18S rRNA) were designed by Assay-on-Demand™ gene expression products (Applied Biosystems, Foster City, CA, U.S.A.). cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Promega, San Luis Obispo, CA, U.S.A.) using 2 μg of total RNA primed with random hexamer primers following the manufacturer's instructions. Real-time PCR was carried out with the ABI PRISM 7500 systems (Applied Biosystems) according to the manufacturer's instructions. PCR amplification of 18S rRNA was done for each sample as a control of sample loading and to allow normalization between samples. The mRNA copy numbers were calculated for each sample by the instrument software using C_t value ("arithmetic fit point analysis for the lightcycler"). Results were expressed in copy numbers, calculated relative to unstimuled cells, after normalization against 18S rRNA, as described (2).

Promoter activity. Tubuloepithelial cells were cultured in six-well plates, 24 h after they were transiently transfected with 2 μg of a promotorless luciferase gene or a luciferase gene containing VEGF promoter (Stu I reporter) (20), in the presence of FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) and 10% FBS. After a 24-h serum starvation step, cells were treated with AngII and $CoCl_2$ for an additional 24 h, and then luciferase/renilla activity was evaluated with a Promega kit.

The 5′ flaking sequence of human, mouse, and rat VEGF genes in this DNA region (Stu I reporter) revealed a high degree of evolutionary conservation. In particular, there is conformity in four sequences. These correspond to the HIF-1 binding site 5′-TACGTGGG (−975 to −968), the AP-1 site 5′-TGACTAA (−937 to −931), the "NF-κB-like" sequence 5′-GGGTTTTGCC (−1,000 to −991), and the sequence 5′-

ACAGGTC (−962 to −956), responsible for hypoxia-induced VEGF promoter activation (20).

The expression vector containing cDNA for the dominant-negative form of c-jun and a mutated form of IκB, which inhibits AP-1 and NF-κB and activation, respectively, were kindly provide by M.A. Iñiguez (Centro de Biologia Molecular Severo Ochoa, Madrid), and they were transfected 1 h before addition of the promoter construction.

Determination of HIF-1

Nuclear extracts. Nuclear extracts were prepared by homogenization and centrifugation as described (2).

HIF-1 DNA-binding activity. HIF-1 DNA-binding activity was determined in nuclear extracts by binding with ^{32}P-labeled double-stranded oligonucleotide probe and analyzed by electrophoretic mobility shift assay (EMSA) electrophoresis. The HIF-1 oligonucleotide sequence (5′-TGCATAGCTGGGCTCCAACAG-3′) contains the HIF-1 DNA-binding site of the VEGF promoter (22). Competition experiments were performed with 100-fold molar excess of unlabeled oligonucleotide.

Western blot. To quantify nuclear HIF-1α levels, western blot analyses were also done. Nuclear proteins (30 μg) were resolved using sodium dodecyl sulfate/6% polyacrylamide gels. After electrophoresis, samples were transferred to membranes, then blocked in buffer [phosphate-buffered saline (PBS) with 0.1% Tween-20, 7.5% dry skimmed milk] for 1 h, and then incubated with a mouse monoclonal HIFα antibody diluted 1:500 (by AbCam, Cambridge, U.K.) overnight at 4°C. After washing, detection was made by incubation with goat anti-mouse peroxidase-conjugated secondary antibody. The protein complexes were visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). In all experiments, protein content was determined by the bicinchoninic acid method. The quality of proteins and efficacy of protein transfer were evaluated by Red Ponceau staining (data not shown).

The autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One, Bio-Rad, Madrid, Spain), obtaining densitometric arbitrary units. Results are expressed as n-fold increase over control in densitometric arbitrary units.

Immunohistochemistry

Paraffin-embedded sections were treated for immunohistochemistry with a marker of oxidative injury of lipids, the 4-hydroxy-2-nonenal (4-HNE) antibody (Oxis Health Products, Portland, OR, U.S.A.) diluted 1:75 in PBS overnight at 4°C in a humid atmosphere. Thereafter, sections were processed with a corresponding secondary anti-IgG peroxidase antibody (Amersham Pharmacia Biotech) at 1:200. Immunostaining was detected with 3,3′-diaminobenzidine (Sigma), and sections were counterstained with Mayer's hematoxylin (Sigma) (6). Negative controls without the primary antibody, or using an unrelated antibody, were included to check for nonspecific staining.

Statistical analysis

Results are expressed as the means ± SEM of the experiments made. Significance was established by the GraphPAD Instat using Student's *t* test (GraphPAD Software), Mann–Whitney test (nonparametric *t* test), and ANOVA nonparametric test (Kruskal–Wallis test), and differences were considered significant if the p value was <0.05.

RESULTS

AngII increases VEGF gene expression in cultured tubular cells

The NRK52E cell line was treated with AngII (10^{-7} mol/L) for increasing times, and VEGF gene expression was determined by real time RT-PCR. In growth-arrested tubular cells, AngII up-regulated VEGF mRNA with a maximum between 3 and 6 h. After at 18 h, the levels of VEGF decreased to control values (Fig. 1A).

The next series of experiments were performed to examine which AngII receptor subtype was involved in VEGF mRNA induction in renal tubular cells. The AT_1 antagonist losartan and/or AT_2 antagonist PD123319 partially diminished AngII-induced VEGF mRNA levels. When both receptors were blocked at the same time, the VEGF overexpression caused by Ang II was completely abolished (Fig. 1B). These results suggest that AngII-induced VEGF up-regulation was mediated by both AT_1 and AT_2 receptors.

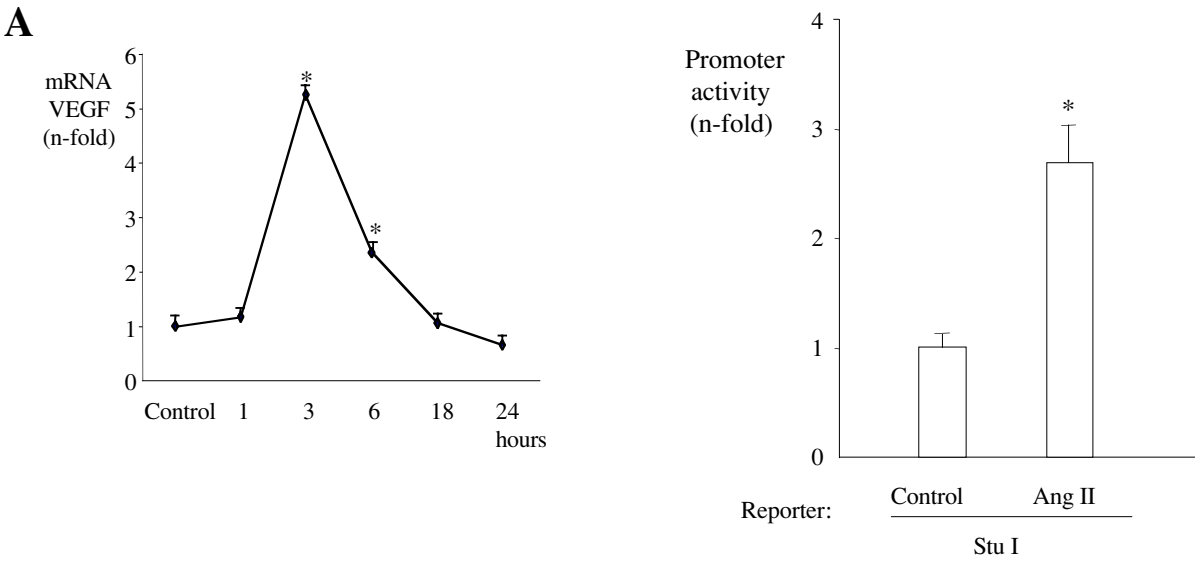
AngII increases VEGF promoter activity in cultured tubular cells

In order to examine whether the induction of VEGF mRNA was due to transcription activation, transient transfection with a reporter vector containing the VEGF promoter associated with luciferase (VEGF-Stu I reporter) in the absence or presence of AngII was made. In AngII-treated tubular cells, the VEGF-Stu I reporter caused 2.6-fold increase versus controls (Fig. 2). Treatment with AngII had minimal effect on the luciferase promotorless construct (data not shown).

In EC, activation of VEGF by AngII was mediated by AP-1 and NF-κB (7). In some experiments, renal tubular cells were cotransfected with a mutant IκB expression vector or a dominant negative of c-jun to block NF-κB or AP-1 activation, respectively. However, there were no changes in AngII-induced VEGF-Stu I reporter activation (data not shown), suggesting that neither NF-κB nor AP-1 are involved in VEGF overexpression caused by AngII in renal tubuloepithelial cells, and indicates that the transcription factors implicated in this AngII action could be tissue-specific.

AngII activates HIF-1α in cultured tubular cells

The promoter of VEGF contains HIF-1 binding sites. We analyzed whether AngII activates the HIF-1 signaling pathway by evaluating HIF-1α nuclear protein levels by western blotting in NRK52E cells. HIF-1α protein expression was undetectable under normal conditions, as previously described



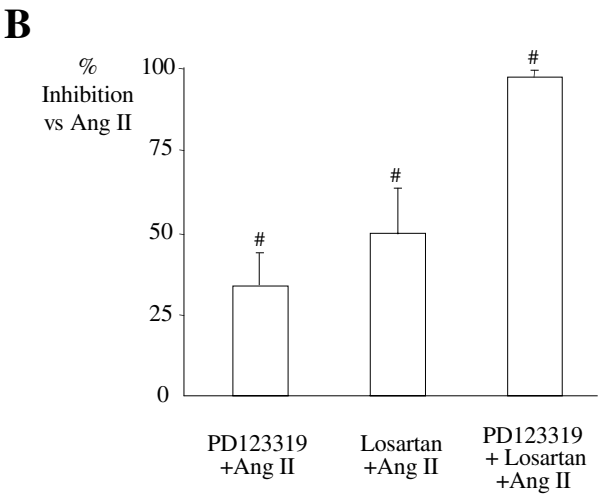


FIG. 1. (A) AngII increases VEGF mRNA expression in cultured tubular cells. Growth-arrested tubular cells were stimulated with 10^{-7} mol/L AngII from 1 to 24 h. (**B**) Effect of AT_1 and AT_2 antagonists on AngII-induced VEGF mRNA expression. Cells were pretreated for 30 min with 10^{-6} mol/L losartan (AT_1 antagonist) or 10^{-5} mol/L PD123319 (AT_2 antagonist), and then stimulated with 10^{-7} mol/L AngII for an additional 6 h. Panels show the means ± SEM of three to five experiments made in triplicate. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus AngII.

FIG. 2. AngII increases VEGF promoter activity in cultured tubular cells. Cells were transiently transfected with a VEGF/luc reporter construct and TK-renilla (internal control), and 24 h later were treated with 10^{-7} mol/L AngII for 24 h. Values are the means ± SEM of four replicates of five independent experiments. $^{*}p < 0.05$ versus control.

(23). However, stimulation with AngII caused induction of HIF-1α protein expression after 2 h, which remained elevated until 6 h (Fig. 3A). Therefore, we evaluated HIF-1 DNA-binding activity by EMSA. AngII increased HIF-1 DNA-binding activity after 4 h, which remained elevated until 6 h (Fig. 3B). The specificity of the binding of the HIF-1 complexes was demonstrated by the complete displacement of the specific bands with an excess of cold oligonucleotide (Fig. 3B).

To investigate whether HIF-1α mediates AngII-induced VEGF expression, NRK52E cells were preincubated with an antisense HIF-1α ODN (5′-CCGGCGCCCTCCAT-3′), which blocks endogenous HIF-1α production, and a sense HIF-1α ODN (5′-ATGGAGGGCGCCGGC-3′) as control (41). The ability of this antisense HIF-1α ODN to suppress both constitutive and hypoxia-induced HIF-1α expression had been checked in previous studies (41). In the presence of antisense HIF-1α ODN, AngII-induced VEGF mRNA overexpression after 6 h was significantly diminished (Fig. 3C), whereas sense HIF-1α ODN did not display any effect (data not shown). These data suggest that HIF-1α has a crucial role in the VEGF up-regulation caused by AngII in renal tubular cells.

Antioxidants decrease AngII-induced VEGF expression and HIF activation

Previous studies have demonstrated that redox mechanisms are involved in the regulation of VEGF in VSMC (24). In tubular cells, AngII can activate ROS production (39). For this reason, we investigated the role of ROS in HIF-1 activation and in the induction of its target gene VEGF caused by AngII in tubuloepithelial cells.

Exposure of tubuloepithelial cells to different antioxidants, such as diphenyleneiodonium (DPI; an inhibitor of flavoprotein-containing enzymes such as NADH/NADPH oxidase), *N*-acetylcysteine (NAC), and 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron; O_2^- scavenger), significantly reduced the stimulatory effect of AngII on VEGF gene expression at 6 h (Fig. 4A). No effect was found when each antioxidant was used alone with respect to control levels (data not shown).

The p22phox is a critical component of the NADH/NADPH oxidase (13), and it is essential for AngII-mediated ROS production (37). Tubuloepithelial cells were preincubated for 1 h with a phosphorothioate-modified p22phox an-

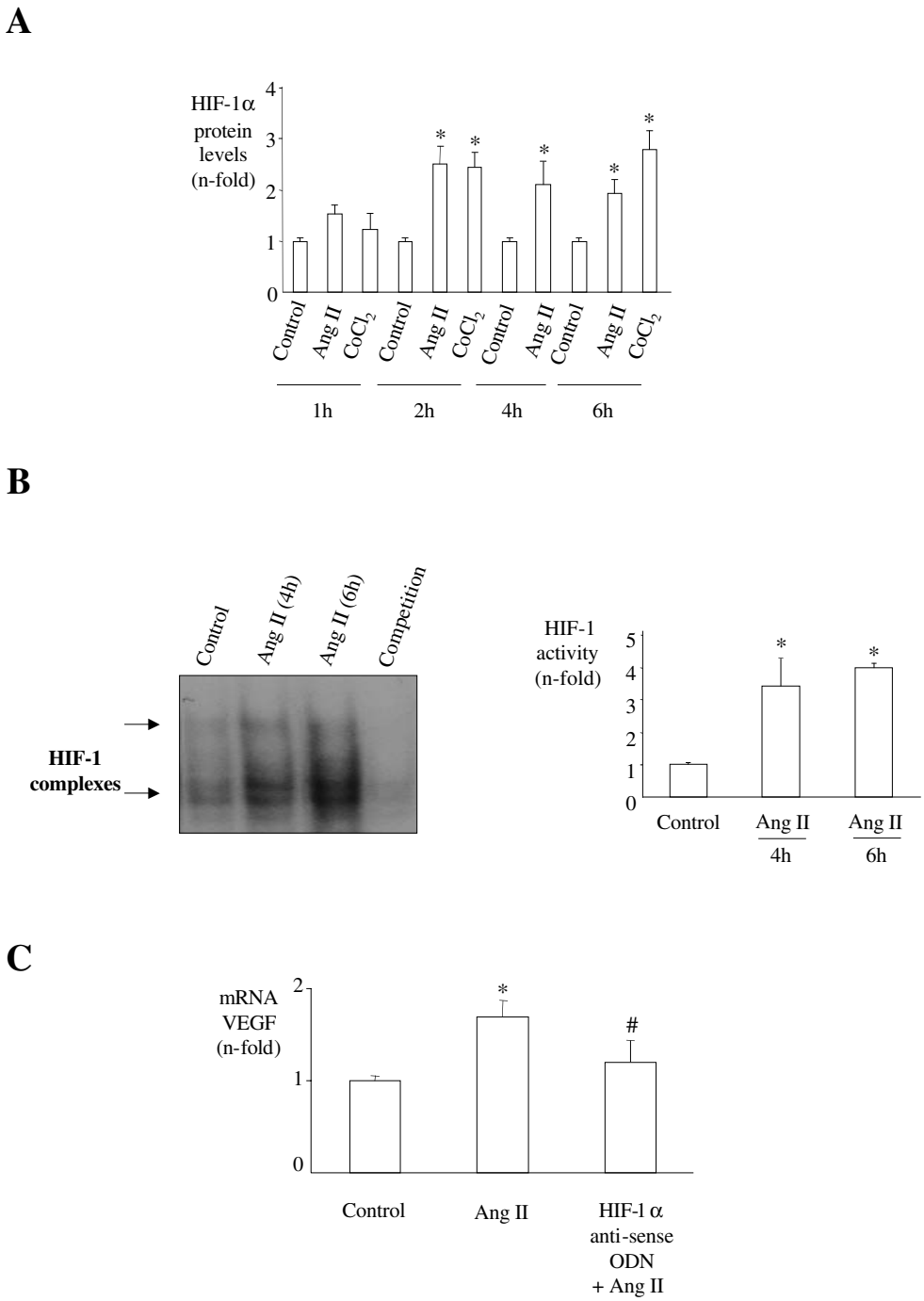


FIG. 3. (A) AngII induces HIF-1 in cultured tubular cells. Cells were incubated with 10^{-7} mol/L AngII from 1 to 6 h. $CoCl_2$ (10^{-4} mol/L) was used as positive control. After the incubation period, HIF-1α nuclear protein levels were determined by western blot. Values are the means ± SEM of five experiments. (**B**) AngII activates the transcription factor HIF-1 in cultured tubular cells. Cells were incubated with 10^{-7} mol/L AngII from 1 to 6 h. After the incubation period, nuclear extracts were isolated, and HIF-1 complex activity was determined by binding assay with a labeled HIF-1 oligonucleotide and analyzed by EMSA. Competition assays with a 100-fold excess of unlabeled HIF-1 oligonucleotide show specific HIF-1 complexes (marked by arrows). The left panel shows a representative EMSA experiment, and the right panel the densitometric analysis of five experiments done (means ± SEM). *$p < 0.05$ versus control. (**C**) HIF-1α mediates AngII-induced VEGF expression in cultured tubular cells. Cells were preincubated for 1 h with HIF-1α antisense ODN (20 μg/ml) added directly to the medium. Then cells were treated with 10^{-7} mol/L AngII for 6 h, and VEGF gene expression was determined by real-time PCR. Values are the means ± SEM of five experiments done. *$p < 0.05$ versus control; #$p < 0.05$ versus AngII.

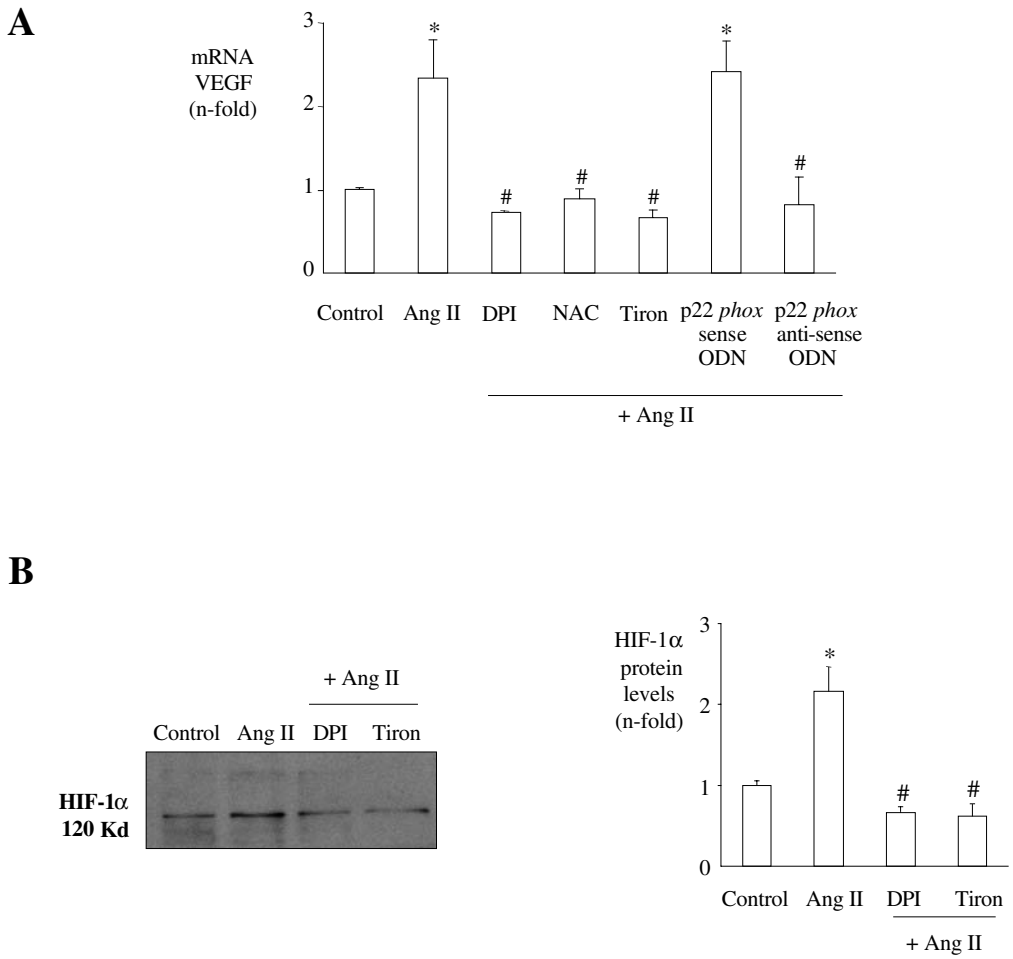


FIG. 4. (A) Antioxidants diminish AngII-induced VEGF expression in cultured tubular cells. Cells were preincubated for 1 h with the antioxidants (DPI, 10^{-5} mol/L; NAC, 2×10^{-4} μmol/L; and Tiron, 5×10^{-3} mol/L), p22phox antisense, or p22phox sense ODNs (20 μg/ml) added directly to medium. Then cells were treated with 10^{-7} mol/L AngII for 6 h. VEGF gene expression was determined by real-time PCR. Values are the means ± SEM of five experiments done. *$p < 0.05$ versus control; #$p < 0.05$ versus AngII. (**B**) Antioxidants diminish AngII-induced HIF-1α protein production in cultured tubular cells. Cells were preincubated for 1 h with the antioxidants and then were treated with 10^{-7} mol/L AngII from 4 h. A representative western blot experiment (left) and data of densitometric analysis (right) of five experiments (means ± SEM) are shown. *$p < 0.05$ versus control; #$p < 0.05$ versus AngII.

tisense ODN (5′-TGCCAGCGCCTGTTCGTTGGC-3′), derived from a consensus sequence of bovine, human, murine, and porcine p22phox genes, or with sense p22phox ODN (5′-GCCAACGAACAGGCGCTGGC-3′) used as control. As shown in Fig. 4A, the presence of antisense p22phox ODN diminished VEGF overexpression mediated by AngII, whereas no effect was seen in the presence of sense p22phox ODN (5′-GCCAACGAACAGGCGCTGGC-3′).

Preincubation with DPI or Tiron diminished AngII-induced HIF-1α nuclear protein (Fig. 4B). Similar results were obtained with the experiments that examined HIF-1α-DNA-binding activity (data not shown). All these data demonstrate that AngII regulates HIF-1 and VEGF by a redox-sensitive mechanism.

AngII activates VEGF, HIF, and the oxidative response in the kidney

To evaluate the *in vivo* effect of AngII in the kidney, a model of systemic infusion of AngII into mice was used. After 3 days, AngII-infused mice presented slightly increased renal expression of VEGF and VEGFR2 (Fig. 5A) compared with controls, whereas VEGFR1 was not modified (data not shown). Treatment with the AT_1 antagonist losartan or with the AT_2 PD123319 diminished VEGF mRNA expression (around 42% and 32% versus AngII-infused mice, respectively). AngII-induced VEGFR2 expression was decreased with losartan.

AngII-infused mice also presented elevated renal HIF-1 DNA-binding activity compared with controls (Fig. 5B). The

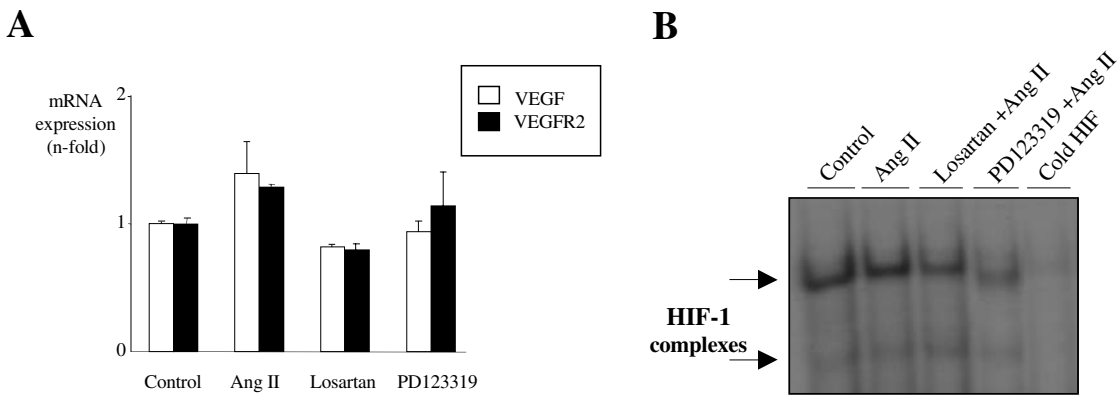


FIG. 5. (A) AngII *in vivo* increases VEGF mRNA expression; role of AT_1 and AT_2 receptors. Animals were treated for 24 h with the specific AT_1 (losartan; 10 mg/kg/day) or AT_2 antagonist (PD123319: 30 mg/kg/day). Then mice were infused with AngII (1,000 ng/kg/min) for 3 days. VEGF and VEGFR2 gene expression was determined by real-time PCR. Values are the means ± SEM of four to six animals in each group. (**B**) AngII *in vivo* activates HIF-1 in the kidney. Animals were infused with AngII (1,000 ng/kg/min) for 3 days. A representative EMSA experiment shows one animal of each group. Specificity of the reaction was demonstrated with a 100-fold excess of unlabeled HIF-1 oligonucleotide.

AngII-induced renal HIF-1 activation was diminished by both AT_1 and AT_2 antagonists.

We evaluated the renal oxidative status after 3 days of AngII infusion. Kidney sections with AngII-infused mice presented a marked increase in the immunostaining of a marker of oxidation (4-HNE) principally in tubules. These results indicated a prooxidant effect of AngII infusion in the kidney (Fig. 6). Both AT_1 and AT_2 antagonists decreased renal 4-HNE immunostaining. All these results show that *in vivo* AngII caused VEGF up-regulation, HIF-1 activation and ROS production in the kidney.

DISCUSSION

In this article, we have investigated the molecular mechanisms involved in VEGF overexpression caused by AngII. We have found that the HIF-1 signaling pathway and redox mechanism are involved in AngII-induced VEGF regulation in the kidney.

Several studies have described that AngII regulates VEGF in the kidney. We have demonstrated that in cultured tubuloepithelial cells (NRK52E cell line), AngII increases VEGF mRNA expression maximally between 3 and 6 h. In murine

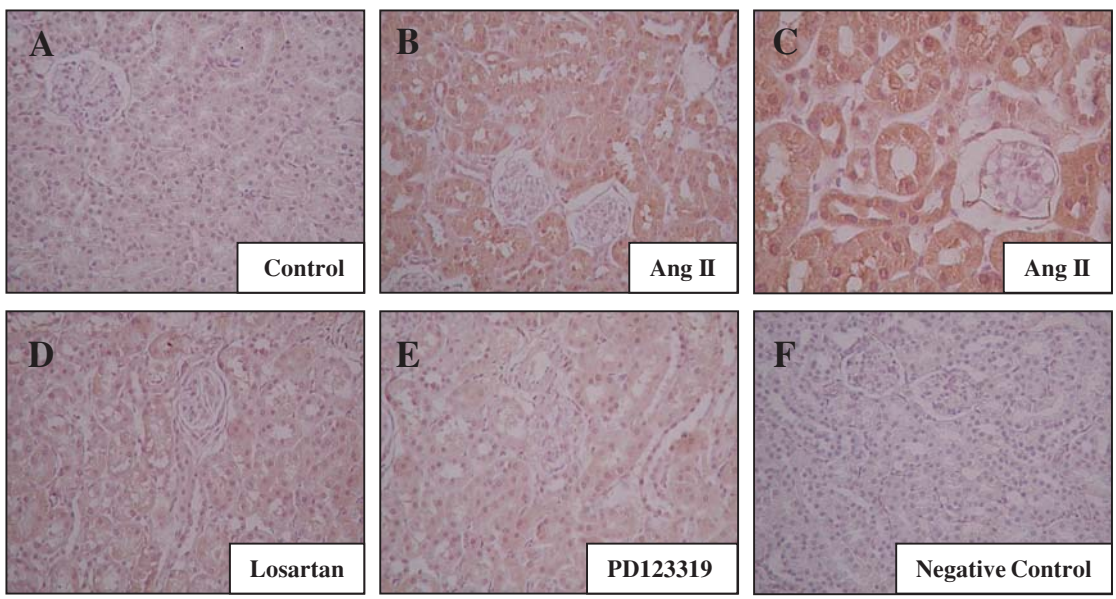


FIG. 6. AngII *in vivo* increases oxidative stress in the kidney. 4-HNE immunostaining was done in mouse kidney. Magnification, ×20 (**A, B, D–F**) and ×40 (**C**).

proximal tubular epithelial cells, a rapid increase in VEGF protein expression by AngII has also been described (10), indicating that in tubular cells of different species AngII up-regulates VEGF. In human mesangial cells, AngII increased VEGF gene expression (13, 27), suggesting that AngII may affect endothelial cell functionality during the development of renal diseases affecting the glomerulus. The AngII receptor involved in VEGF gene regulation is not clearly established. Several studies in different tissues have shown that AngII-induced VEGF up-regulation was mediated by AT_1 receptors (7, 14). In this sense, in human mesangial cells AngII regulates VEGF gene through AT_1 activation (13, 27). However, in cultured tubuloepithelial cells, both AT_1 and AT_2 receptors participate in VEGF gene expression, as we have shown by specific receptor antagonists. The effect of *in vivo* blockade of AngII on VEGF regulation is still controversial. Divergent effects of angiotensin-converting enzyme (ACE) or AT_1 antagonists have been reported. ACE inhibition was associated with increased VEGF expression in the model of remnant kidney (18). Treatment with AT_1 antagonists caused a significant reduction in VEGF expression in podocytes of diabetic rats (19) and in cyclosporine nephropathy (34). We have found that *in vivo*, in the model of systemic infusion of AngII into mice, both AT_1 and AT_2 antagonists diminished VEGF gene overexpression. The role of VEGF in renal pathophysiology is really complex. VEGF is deleterious in some pathological settings, but may contribute to accelerate the recovery in other renal diseases (1, 8, 12, 16–19, 23, 33, 34). VEGFR2 is the most relevant VEGF receptor in functional terms, and it has been implicated in renal pathology (16, 33). In AngII-infused mice, VEGFR2 up-regulation was blocked by AT_1 antagonists. All these data show that future studies are needed to define clearly the functional importance of VEGF in AngII-induced renal damage.

We have demonstrated that in cultured tubuloepithelial cells AngII increases VEGF mRNA and promoter activation, showing that AngII regulates VEGF at the transcription level. We have further investigated the mechanisms involved in VEGF regulation, studying the role of transcription factor activation. AngII activates several transcription factors, such as AP-1 and NF-κB, involved in renal damage (30, 31). The promoter of VEGF contains AP-1 and NF-κB binding sites, but we have observed that transfection with mutant IκB or c-jun dominant-negative expression vectors, to block NF-κB or AP-1 activation, respectively, did not modify AngII-induced VEGF promoter activation in renal tubuloepithelial cells. The promoter of VEGF also contains HIF-1 binding sites. However, whether AngII activates the HIF-1 signaling pathway in the kidney has not been investigated. We have shown here that in renal tubuloepithelial cells AngII induced HIF-1α protein levels, increased HIF-1 DNA-binding activity, and enhanced reporter gene activity of constructs containing HIF-1 regions of the VEGF promoter. Moreover, an antisense HIF-1α oligonucleotide significantly diminished AngII-induced VEGF gene expression. Finally, systemic infusion of AngII into mice caused VEGF gene overexpression and elevated HIF-1 DNA-binding activity in the kidney. The AngII-induced renal HIF-1 activation was diminished by both AT_1 and AT_2 antagonists. Our results suggest that activation of the transcription factor HIF-1 could represent a novel mechanism by which AngII regulates VEGF gene expression in the kidney.

One of the most important mechanisms involved in AngII-induced renal damage is the production of ROS, which can act as intracellular signaling molecules (31, 36). AngII can induce ROS production in different cell types, including renal tubular cells (36). ROS activate multiple intracellular proteins, enzymes, transcription factors, including HIF-1 (31, 36), and genes, such as VEGF (28). We examined the effect of DPI, a potent inhibitor of flavonoid-containing enzymes, such as NADPH oxidase. DPI decreased AngII stimulation of VEGF expression in tubuloepithelial cells, suggesting the involvement of a redox mechanism in the regulation of VEGF. The role of p22phox-containing NADPH oxidase has also been confirmed using a p22phox antisense oligonucleotide. Other antioxidants, such as NAC and Tiron, significantly diminished AngII-induced VEGF overexpression observed in these cells. All these data clearly indicate that VEGF induction caused by AngII is regulated by a redox-mediated mechanism. In these cells, we have observed that the up-regulation of HIF-1α protein and HIF-1 DNA-binding activity caused by AngII was significantly attenuated by several antioxidants. These findings demonstrate that the redox-sensitive cascade activated by ROS, derived from the p22phox-containing NADPH oxidase, is crucially involved in the HIF-1 signaling pathway stimulated by AngII. These results were confirmed *in vivo*. We have found that AngII-infused mice presented renal oxidative stress, HIF-1 activation, and VEGF overexpression, indicating that *in vivo* regulation of VEGF could be mediated by HIF signaling in a redox-sensitive process.

Other studies support our findings in vascular diseases. Vascular remodeling is associated with oxidative stress, hypoxia, and enhanced levels of VEGF. AngII infusion to mice increased aortic expression of VEGF and its receptors and caused aortic inflammation (monocyte infiltration) and remodeling (wall thickening and fibrosis). The blockade of VEGF by the soluble VEGFR1 (sFlt-1) gene transfer did attenuate the AngII-induced inflammation and remodeling. In contrast, sFlt-1 gene transfer did not affect AngII-induced arterial hypertension and cardiac hypertrophy (42).

In conclusion, our data show that AngII *in vivo* and *in vitro* up-regulates renal VEGF expression by a mechanism that involves HIF-1 activation. Inhibition of AngII-induced VEGF expression and HIF-1 activation by the antioxidants indicated redox-sensitive mechanisms involved in this signaling pathway in the kidney.

Perspective section

As the VEGF system is affected in a wide variety of kidney diseases, interventions to manipulate VEGF could be promising therapeutic tools. At this moment, there are several strategies to target VEGF and its receptors. So far, the experience with these treatments in renal disease is limited to some animal models. Interference with the renin–angiotensin system and ROS signaling could be an indirect tool to modulate the VEGF-mediated effects. Future studies are necessary to know the functional importance of VEGF in the mechanisms of AngII- and/or ROS-induced renal damage in the kidney.

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ABBREVIATIONS

ACE, angiotensin-converting enzyme; AngII, angiotensin II; AP-1, activator protein-1; AT_1 and AT_2, angiotensin II receptor types 1 and 2; DPI, diphenyleneiodonium chloride; EC, endothelial cell; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; HIF-1, hypoxia-inducible factor-1; 4-HNE, 4-hydroxy-2-nonenal; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; NRK52E, normal rat kidney tubular epithelial cell 52E; ODN, oligodeoxynucleotide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcriptase–polymerase chain reaction; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid; VEGF, vascular endothelial growth factor; VEGFR1, VEGFR2, and VEGFR3, vascular endothelial growth factor receptors 1, 2, and 3; VSMC, vascular smooth muscle cells.

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