Increased Renal Vascular Endothelial Growth Factor and Angiopoietins by Angiotensin II Infusion Is Mediated by Both AT_1 and AT_2 Receptors

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Abstract. A link between angiotensin II and cell proliferation has previously been reported. However, there remains controversy as to the role of the individual angiotensin II receptor subtypes in mediating these effects and their link to angiogenic cytokines and their receptors. Male Sprague-Dawley rats were infused with either angiotensin II or vehicle for 14 d at a dose of 58.3 ng/min. Angiotensin II–infused rats received no treatment, an AT_1 receptor antagonist valsartan (30 mg/kg per d), or an AT_2 receptor antagonist PD123319 (830 ng/min). Gene expression of vascular endothelial growth factor (VEGF) and receptor VEGF-R2, as well as Tie-2 and its ligands angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) were assessed by reverse transcription–PCR. Protein expression was assessed by Western blotting and immunohistochemistry. Gene and protein expression of VEGF, Ang-1, and Ang-2 were increased by angiotensin II infusion. Valsartan and PD123319 attenuated angiotensin II–associated increases in VEGF gene and protein expression. Ang-1 and Ang-2 gene but not protein expression were reduced by both treatments. These changes occurred in the context of attenuation of angiotensin II–induced glomerular cell proliferation by both valsartan and PD123319. *In situ* hybridization and immunohistochemical studies localized VEGF, Ang-1, and Ang-2 expression to the epithelial cells of the glomerulus, and VEGF-R2 and Tie-2 receptors to the endothelial cells of the kidney. These findings extend the increasing evidence that the AT_2 receptor, in addition to the AT_1 receptor subtype, plays an important role in mediating the proliferative actions of angiotensin II in the kidney.

Cellular proliferation is considered to be an important part of the renal and specifically the glomerular response to various stimuli and a range of primarily angiogenic cytokines and their receptors (1). The endothelial receptor tyrosine kinases and their ligands are known to have unique roles in the initiation and maintenance of growth within the vasculature. These include vascular endothelial growth factor (VEGF; which acts predominantly through one of its receptors [VEGF-R2] (2,3)), in combination with the Tie-2 ligands, angiopoietin 1 (Ang-1) and angiopoietin 2 (Ang-2) (2,4).

Hanahan *et al.* (5) proposed that these factors worked in synchrony in the angiogenic response. First, binding of Ang-2 is vital for loosening the vasculature and allowing the penetration of angiogenic cytokines such as VEGF, which then initiates the proliferation and migration of endothelial cells, resulting in the formation of new blood vessels (6). Binding of Ang-1 is then needed to recruit stromal cells that are required to encase and stabilize these primitive endothelial tubes (7,8). This hypothesis has since been supported in a number of models (9–11). However, the initiating stimuli to activate these pathways, particularly within the kidney, remain uncertain.

Recent studies have demonstrated increased evidence of angiotensin II involvement in the regulation of these angiogenic cytokines and their receptors. Exposure of angiotensin II to cardiac microendothelial cells (CMEC) was associated with an elevation in Ang-2 and VEGF mRNA expression (12). This response was confirmed in cultured bovine retinal endothelial cells, where Ang-2 and VEGF were elevated in response to angiotensin II exposure (3,4). Angiotensin II had previously been reported to potentiate VEGF-mediated angiogenesis in the same cells via upregulation of VEGF-R2 (13).

Clearly, angiotensin II plays a role in promoting angiogenesis. However the relative roles of the different angiotensin II receptor subtypes in mediating these effects are largely unknown. At least two angiotensin receptor subtypes have been identified, the type 1 (AT_1) and the type 2 (AT_2) receptor. The functions of the AT_1 receptor have been investigated extensively, and this receptor is considered to be proangiogenic. By contrast, the function of the AT_2 receptor in angiogenesis remains to be ascertained. The AT_2 receptor is mainly expressed in fetal tissues (14) and neonatal kidneys (15–17) and has been considered to play its major role during kidney organogenesis. In the adult, however, the AT_2 receptor is expressed at very low levels, particularly in kidney (18,19) but also in the heart (15,20) and vasculature. There is increasing evidence that the AT_2 receptor plays a pivotal role in the

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pathogenesis of vascular injury by mediating a range of angiotensin II–mediated effects, including effects on vasoreactivity, cytokine expression, cellular proliferation, and apoptosis (21–25). It remains to be determined whether the AT_2 receptor participates in the effects of angiotensin II on angiogenic cytokines such as VEGF and the angiopoietins as well as their respective receptors.

The aims of the present study were, first, to explore the *in vivo* effects of angiotensin II on expression of these angiogenic cytokines in the kidney and, second, to study the relative roles of the AT_1 receptor and AT_2 receptor subtype on these angiogenic growth factors and their receptors.

Materials and Methods

Rat Model

Adult male Sprague-Dawley rats, housed at the Biologic Research Laboratory of the Austin and Repatriation Medical Centre, were used in this study. The protocols for animal experimentation and the handling of animals were in accordance with the principles established by the Animal Ethics Committee of the Austin and Repatriation Medical Centre.

Sixty rats were randomly allocated into six groups ($n = 10$/group) and treated for 14 d. Group 1 received a vehicle infusion of 0.15 mol/L sodium chloride and 1 mmol/L acetic acid via an Alzet osmotic minipump (Model 2002; Alzet Corp., Cupertino, CA) implanted subcutaneously in the midscapular region. Animals were anesthetized with enflurane (Ethrane; Abbott Australasia, Kurnal, NSW, Australia). Group 2 received an infusion of human angiotensin II (Auspep; Melbourne, Victoria, Australia) at a concentration of 7 mg/ml. On the basis of the pumping rate of the minipumps (0.5 μl/h), angiotensin II was administered at a dose of 58.3 ng/min, a dose shown to induce hypertension and vascular hypertrophy (26). Group 3 received the angiotensin II infusion plus valsartan (provided by Dr. Marc de Gasparo, Novartis, Basel, Switzerland) at a dose of 30 mg/kg per d by daily gavage. Group 4 received an infusion of angiotensin II subcutaneously and was treated with PD123319 (provided by Joan Keiser, Parke-Davis, Ann Arbor, MI) via intraperitoneally implanted Alzet minipump (Model 2002). PD123319 was dissolved at a concentration of 100 mg/ml with sterile water. On the basis of the pumping rate, PD123319 was administered at 830 ng/min. Valsartan and PD123319 were also administered to vehicle-infused rats, respectively (groups 5 and 6). The doses of valsartan and PD123319 used in this study have previously been shown to block AT_1 or AT_2 receptor binding, respectively, as assessed by autoradiography with no evidence that either angiotensin II antagonist affected the other receptor subtype in the kidney (24,27).

Animals were anesthetized by intravenous injection of pentobarbitone sodium at a dose of 60 mg/kg body wt (Roche Molecular Biochemicals, Indianapolis, IN). A midline incision of the abdomen was made, and the kidney was removed and frozen for extraction of RNA/protein for subsequent assessment of gene/protein expression. The remaining kidney was then immersion-fixed in 4% paraformaldehyde and then embedded in wax for subsequent immunohistochemistry and *in situ* hybridization.

Systolic BP (SBP) was measured by indirect tail-cuff plethysmography in prewarmed, unanesthetized animals as described previously (28). Blood samples were collected from the tail vein of conscious rats before the animals were killed for the measurement of plasma renin activity (PRA) (29).

Reverse Transcription–PCR

Three micrograms of total RNA extracted from each kidney was used to synthesis cDNA with the Superscript First strand synthesis system for reverse transcription–PCR (RT-PCR; Life Technologies BRL, Grand Island, NY). VEGF, VEGF-R2, Ang-1, Ang-2, and Tie-2 gene expression was analyzed by real-time quantitative RT-PCR performed with the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, Foster City, CA) (30). Fluorescence for each cycle was quantitatively analyzed by an ABI Prism 7700 Sequence Detection System (Perkin-Elmer, PE Biosystems, Foster City, CA). For controlling for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S ribosomal RNA (rRNA; 18S rRNA TaqMan Control Reagent kit; ABI Prism 7700; Perkin-Elmer). Primers and TaqMan probes for VEGF, VEGF-R2, Ang-1, Ang-2, Tie-2 and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700). For amplification of the VEGF cDNA, the forward primer was 5'-GCGGGCTGCTGCAATG-3' and the reverse primer was 5'-TGCAACGCGAGTCTGTGTTT-3'. The probe specific for VEGF was FAM-5'-TGCCCACGTCGGAGAGCAACGT-3'-TAMRA;FAM = 6-carboxyfluorescein, TAMRA (quencher) = 6-carboxy-tetramethylrhodamine. For the VEGF-R2 cDNA, the forward primer was 5'-CCACTTCTGTCTTGCCACACA-3' and the reverse primer was 5'-CCAACCAATTAAGACCTTCTG-3'. The probe specific to VEGF-R2 was FAM-5'-CCCTCCCCAGTGCTCAGTATTTTAGCTTTG-3'-TAMRA. The forward primer for Ang-1 was 5'-AGATACAACAGAATGCGGTTCAAA-3' and the reverse primer was 5'-TGAGACAAGAGGCTGGTTCCTAT-3'. The specific probe for Ang-1 was FAM-5'-CCACACGGCCACCATGCTGG-3'-TAMRA. The forward primer for Ang-2 cDNA was 5'-GCTGGGCAACGAGTTTGTCT-3' and the reverse primer was 5'-CAGTCCTTCAGCTGGATCTTCA-3'. The probe for Ang-2 was FAM-5'-CTGACCAGTGGGCATCGCTACGTG-3'-TAMRA. The forward primer for the Tie-2 receptor was 5'-CTGAGAACAACATAGGATCAAGCAA-3' and the reverse primer was 5'-TTTCCCCCTCCAAGGTCTTT-3'. The probe for the Tie-2 receptor was FAM-5'-CCCAAGAAATTAGGACACTTCCAGCCCC-3'-TAMRA. The amplification was performed with the following time course: 50°C, 2 min and 10 min at 95°C; and 40 cycles of 94°C, 20 s, 60°C, 1 min. Each sample was tested in triplicate. Results were expressed relative to control kidneys, which were arbitrarily assigned a value of 1.

In Situ Hybridization

Sense and antisense RNA probes for VEGF and VEGFR-2 were generated by *in vitro* transcription (Promega, Madison, WI), as described previously (31). In brief, 4-micron sections were cut from paraformaldehyde-fixed, paraffin-embedded kidney tissue onto 3-aminopropyltriethoxysilane slides and left overnight at 37°C. Tissue sections were dewaxed in histolene, rehydrated and equilibrated in P buffer (50 mmol/L Tris-HCl [pH 7.5], 5 mmol/L EDTA), and incubated in 125 μg/ml Pronase E (Sigma Chemical Co., St. Louis, MO). Sections were then washed and briefly refixed in 4% paraformaldehyde rinsed in milliQ water, dehydrated in 70% ethanol, and air-dried.

Hybridization buffer containing 2×10^4 cpm/μl riboprobe in 300 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L Na_2HPO_4, 5 mmol/L EDTA (pH 8.0), 1× Denhardt's solution, 50% formamide, 17 mg/ml yeast RNA, and 10% wt/vol dextran sulfate was heated to 85°C for 5 min. A 25-μl aliquot of this solution was then applied to each tissue section under a coverslip. Hybridization of tissue to the

riboprobe was performed overnight in humidified chambers. Sense probes for VEGF and its receptor were used on an additional set of tissue sections as controls for nonspecific binding.

After hybridization, slides were washed and coverslips were removed. Sections were then washed and rinsed in RNAse buffer (10 mmol/L Tris-HCl [pH 7.5], 1 mmol/EDTA [pH 8.0], 0.5 mol/L NaCl) and then incubated with RNAse A (150 μg/ml) for 1 h. Sections were later washed, dehydrated in graded ethanol, air-dried, and exposed to Kodak X-Omat autoradiographic film (Kodak, Rochester, NY) for 1 to 3 d. Slides were then dipped in Ilford K5 nuclear emulsion (Ilford, Mobberly, UK) and stored in a light-free box, followed by fixation with Ilford Hypam. Sections were then stained with hematoxylin and eosin for examination under light microscopy.

Immunohistochemistry

Four-micron kidney sections were cut, dehydrated, and used for immunohistochemistry. Sections were pretreated with either trypsin (1.0 mg/ml in 1 M $CaCl_2$) or pepsin (0.1 mg/ml in 0.5 M acetic acid) for 10 to 25 min depending on the primary antibody. Endogenous peroxidase was quenched using 3% hydrogen peroxidase in methanol for 20 min and then blocked with an appropriate serum for 20 min. The sections were then incubated overnight at 4°C with anti-VEGF, VEGF-R2, Ang-1, Ang-2, and Tie-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). A biotinylated antibody (Vector Laboratories, Burlingame, CA) was used as a secondary antibody detected by reaction with horseradish peroxidase–streptavidin–biotin complex and then subsequently with 3,3'diaminobenzidine tetrachloride (DAB; Sigma Chemical Co).

For assessment of cell proliferation, kidney sections were incubated with a monoclonal antibody to proliferating cell nuclear antigen (PCNA; PC-10, DAKO A/S, Copenhagen, Denmark). Biotinylated multilink swine anti-goat, mouse, rabbit immunoglobulin (DAKO Corporation, Carpinteria, CA) was used as a second antibody, followed by horseradish peroxidase–conjugated streptavidin. Peroxidase activity was identified by reaction with DAB substrate. The number of cells positively stained with PCNA were counted manually in the glomeruli and expressed as the PCNA-positive cell number per total glomerular cell number under a light microscope in a masked manner (×400) using an Imaging Analysis System (Imaging Research, St. Catherines, Ontario, Canada) associated with a video camera and a computer.

Western Blotting

Snap-frozen kidneys were homogenized in neutral salt buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 5 mM EDTA) containing protease inhibitors leupeptin (10 μg/ml), PMSF (1 mM), and aprotinin (1 μg/ml). Homogenates were collected and centrifuged at 400 × g at 4°C for 7 min; the supernatant was then removed and ultracentrifuged at 75,000 × g for 1 h. The supernatant was collected, and protein content was measured using the BCA protein assay (Perbio Science UK Ltd., Cheshire, UK).

For Western blotting, 30 μg of each sample was incubated at 65°C for 10 min and separated on a 10% SDS–polyacrylamide electrophoresis gel. Using a semidry transfer tank (Bio-Rad Laboratories, Hercules, CA), proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). Nonspecific binding sites were blocked by overnight incubation with 5% (wt/vol) nonfat milk powder in TBS followed by 1-h incubations of primary and secondary antibodies (Santa Cruz Biotechnology). Bound antibodies were amplified using an avidin-biotin complex and detected by reaction with DAB. The band intensity was quantified using a microcomputer imaging device and expressed as (Density × Area) − Background. Variations in protein loading were detected using α-tubulin (Sigma Chemical Co.), which was run with each gel.

Statistical Analyses

Data are shown as mean ± SEM. Comparisons of group means were performed by Fisher's least significant difference method. $P < 0.05$ was viewed as statistically significant.

Results

Systolic BP

Infusion of angiotensin II was associated with an increase in SBP relative to the vehicle-infused group (AII 212 ± 3 mmHg *versus* untreated 128 ± 2 mmHg; $P < 0.01$, day 13). Treatment of the angiotensin II–infused animals with valsartan attenuated the angiotensin II–induced elevation in BP (AII + Val 139 ± 2 mmHg; $P < 0.01$ *versus* AII, day 13). Treatment of the angiotensin II–infused animals with PD123319 had no effect on BP (AII + PD 211 ± 3 mmHg, day 13). Treatment of the vehicle–infused animals with PD123319 had no effect on SBP (120 ± 4 mmHg, day 13), whereas valsartan reduced SBP (106 ± 3 mmHg, day 13; $P < 0.05$ *versus* vehicle, day 13).

Effect of Angiotensin II on Glomerular Proliferation

Infusion of angiotensin II resulted in an approximately 100% increase in PCNA staining in the glomerulus (Figure 1). This proliferative response was abolished with valsartan treatment and more modestly reduced with PD123319. Treatment of the vehicle-infused animals with either valsartan or PD123319 had no significant effect on glomerular proliferation.

Effect of Angiotensin II Infusion on Gene Expression

Angiotensin II infusion significantly increased the level of VEGF mRNA by approximately 70% (Figure 2A), and this

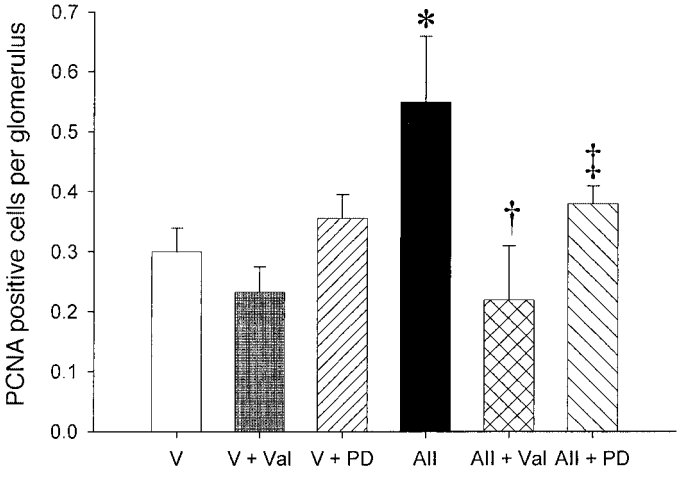

Figure 1. Proliferating cell nuclear antigen (PCNA)-positive cells in the glomeruli after vehicle treatment (V), co-administration of vehicle with valsartan (V + Val) or with PD123319 (V + PD), angiotensin II infusion (AII), or co-administration of angiotensin II with valsartan (AII + Val) or PD123319 (AII + PD). Data are shown as mean ± SEM. *$P < 0.01$ *versus* vehicle; †$P < 0.05$, ‡$P < 0.01$ *versus* AII.

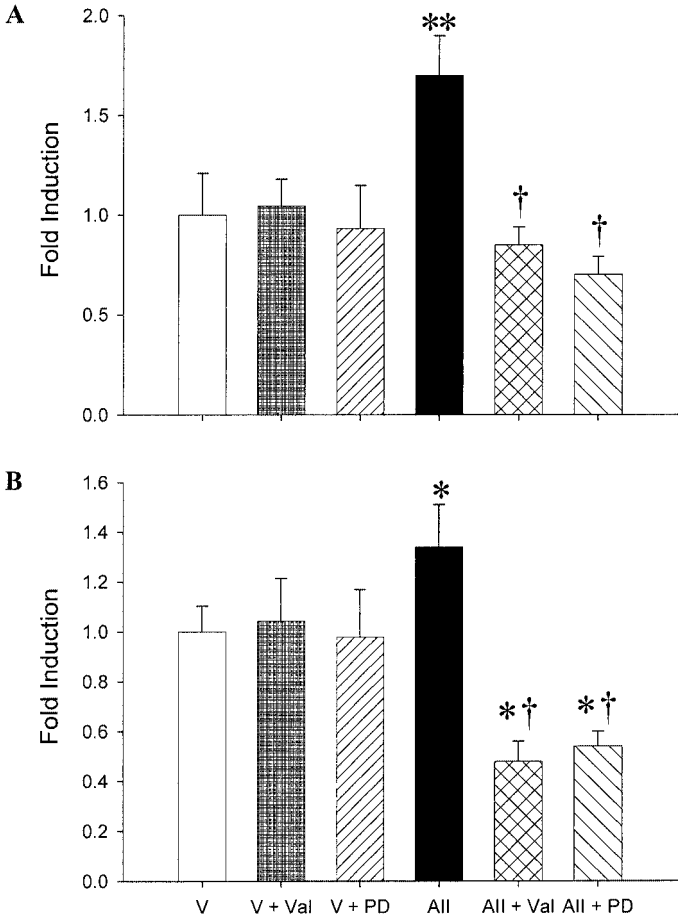

Figure 2. Fold induction of vascular endothelial growth factor (VEGF; A) and VEGF receptor (B) mRNA by reverse transcription–PCR (RT-PCR). V, vehicle; V + Val, vehicle + valsartan; V + PD, vehicle + PD123319; AII, angiotensin II infusion; AII + Val, angiotensin II + valsartan; AII + PD, angiotensin II infusion + PD123319. Data are shown as mean ± SEM. $*P < 0.05$, $**P < 0.01$, *versus* vehicle; $\dagger P < 0.01$ *versus* AII.

increase was ameliorated by treatment with either valsartan or PD123319. VEGF-R2 gene expression (Figure 2B) was increased by 40% with angiotensin II. Treatment of the angiotensin II–infused rats with either valsartan or PD123319 was associated with a significant downregulation of VEGF-R2 gene expression to a level lower that that seen in vehicle alone. Co-administration of the vehicle-infused animals with valsartan or PD123319 had no effect on the gene expression of VEGF or VEGF-R2.

Similarly, administration of angiotensin II upregulated the gene expression of Tie-2 and its ligands. Ang-1 mRNA (Figure 3A) was increased greater than twofold. This upregulation was prevented by treatment with either valsartan or PD123319. Similarly, Ang-2 (Figure 3B) was elevated by approximately 100% and reduced with both valsartan and PD123319. The Tie-2 receptor (Figure 3C) was also upregulated by approximately 80% and attenuated by treatment with either valsartan or PD123319. Treatment of the vehicle-infused animals with either valsartan or PD123319 had no effect on the gene expression of Ang-1, Ang-2, or Tie-2.

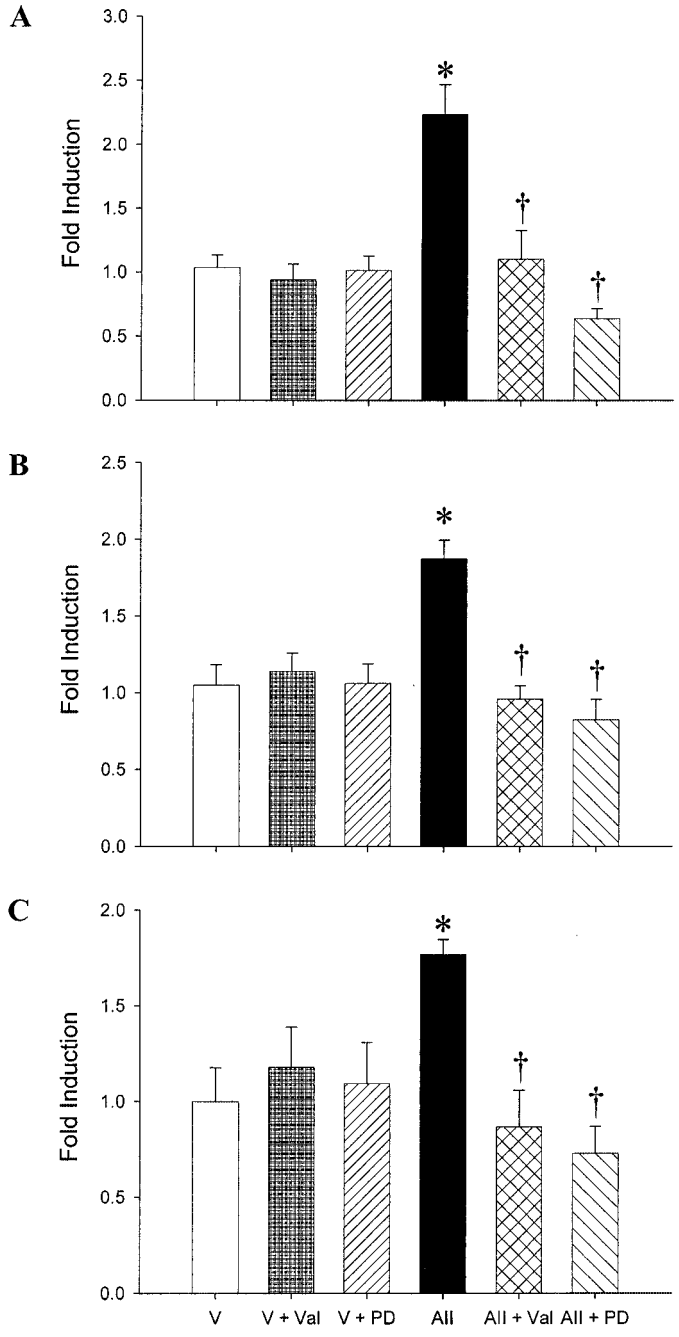

Figure 3. Fold induction of Ang-1 (A), Ang-2 (B), and Tie-2 (C) mRNA by RT-PCR. Data are shown as mean ± SEM. $*P < 0.01$ *versus* vehicle; $\dagger P < 0.01$ *versus* AII.

In situ hybridization localized the major site of VEGF mRNA expression to cells resembling the visceral epithelial cells of the glomerulus. VEGF-R2 mRNA was localized to the endothelial cells of the glomerulus (Figure 4).

Effect of Angiotensin II Infusion on Protein Expression

Immunohistochemistry confirmed that the major site of VEGF protein expression (Figure 5) was the epithelial cells of the glomerulus. Angiotensin II infusion was associated with an elevation in VEGF protein expression as assessed by the in-

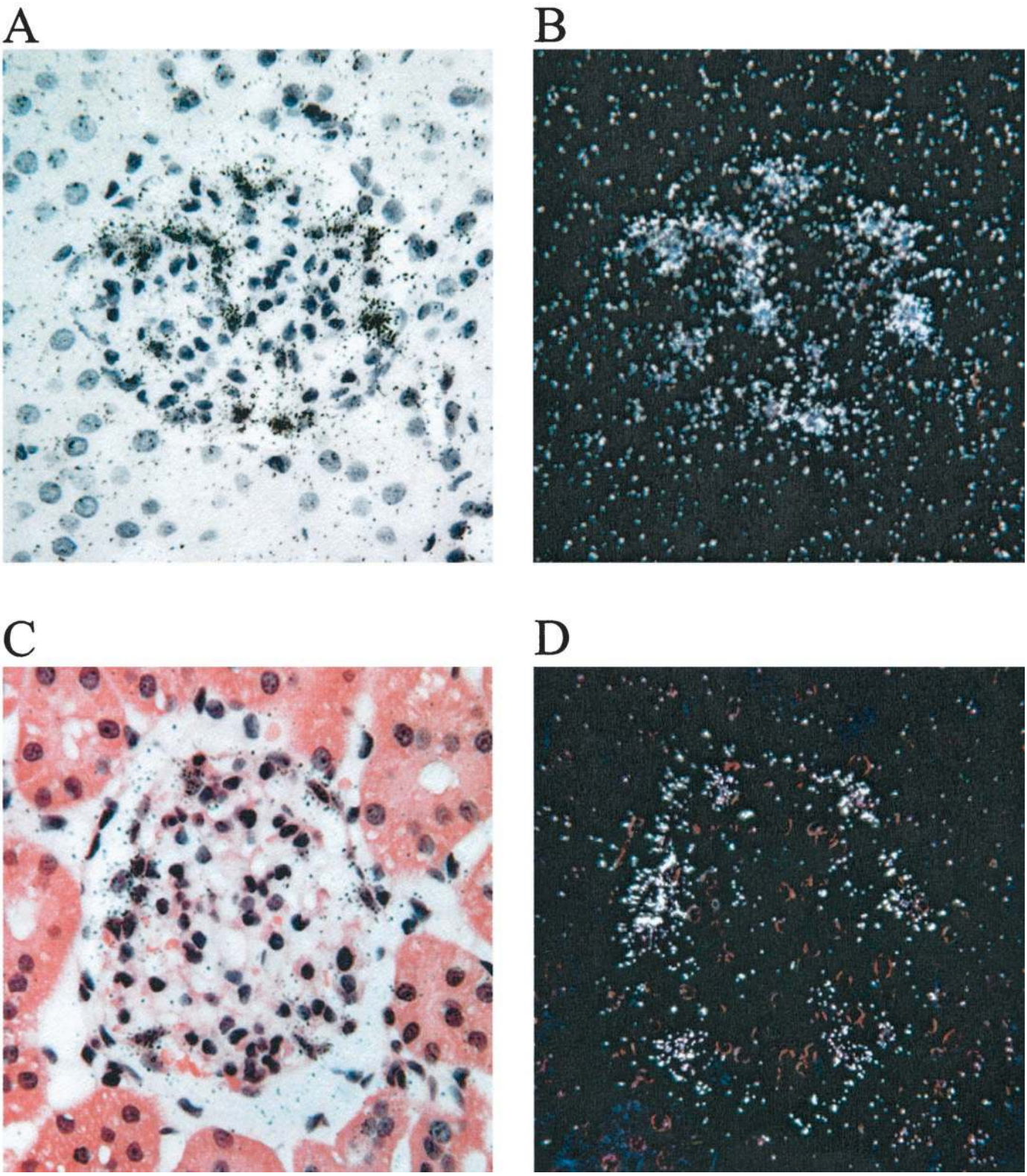

Figure 4. Photomicrographs of representative *in situ* of control animals for VEGF (bright field, A; dark field, B) and VEGF receptor (bright field, C; dark field, D). Magnification, ×400.

crease in glomerular immunostaining (Figure 5B). Treatment with valsartan (Figure 5C) and PD123319 (Figure 5D) reduced the immunostaining to a level similar to that observed in the vehicle group (Figure 5A).

By immunoblotting, VEGF protein was sized at 46 kD. The trend observed with immunohistochemistry was confirmed with quantitative Western blotting. Angiotensin II infusion significantly increased VEGF protein expression by at least

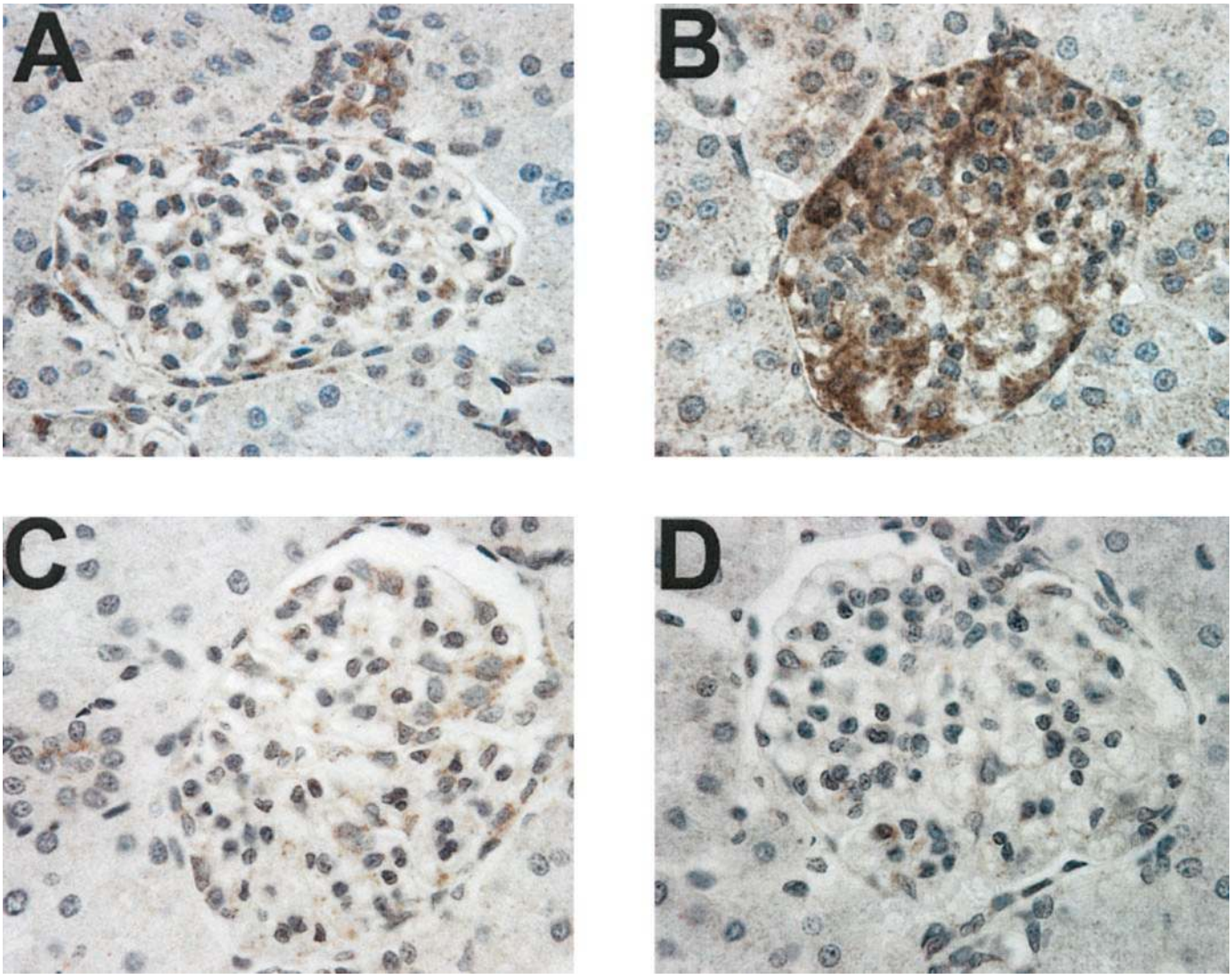

Figure 5. Photomicrographs of VEGF protein for vehicle (A), angiotensin II–infused (B), angiotensin II + valsartan (C), and angiotensin II + PD123319–treated (D) animals by immunohistochemistry. Magnification, ×400.

threefold (Figure 6). This increase was attenuated by valsartan and totally prevented by PD123319 treatment.

By immunohistochemistry, both the Tie-2 ligands, Ang-1 and Ang-2 were located in the epithelial cells of the glomerulus, collecting ducts, and vascular bundles (Figure 7). By immunoblotting, the angiopoietins were sized at approximately 50 kD (Figure 8A). However, no discernible difference could be detected for either angiopoietin among the various groups by immunohistochemistry (Figure 7). Quantitative Western blot analysis revealed that angiotensin II significantly elevated the amount of Ang-1 protein by approximately twofold (Figure 8). This increase was attenuated modestly by valsartan but not by PD123319. Similarly, Ang-2 protein expression was increased by 80% with angiotensin II infusion. Treatment with either valsartan or PD123319 did not attenuate the angiotensin II–induced increase in Ang-2 protein expression. Treatment of the vehicle-infused animals with either valsartan or PD123319 had no effect on the protein expression of Ang-1 or Ang-2 (Figure 9).

Immunohistochemistry localized the VEGF-R2 (Figure 10D) and Tie-2 receptor (Figure 10A) to the endothelial cells of the glomerulus as well as to the endothelial cells of medullary and cortical blood vessels (Figure 10).

Discussion

In the present study, angiotensin II induced an elevation in BP that was associated with an upregulation of both gene and protein expression of VEGF, Ang-1, and Ang-2. Treatment with valsartan and PD123319 ameliorated angiotensin II–associated increases in VEGF gene and protein expression and also Ang-1 and Ang-2 gene expression. These changes occurred in the context of retardation of angiotensin II–induced glomerular cell proliferation by both valsartan and PD123319. *In situ* hybridization and immunohistochemical studies localized VEGF, Ang-1, and Ang-2 expression to the epithelial cells of the glomerulus, and the VEGF-R2 and Tie-2 receptors to the endothelial cells of the kidney. The expression of these angio-

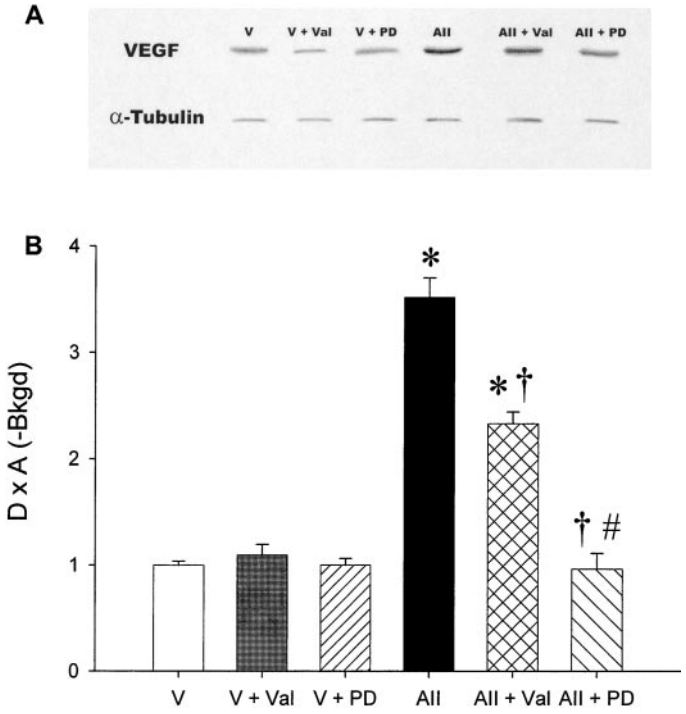

Figure 6. Photograph of a stained nitrocellulose membrane for VEGF protein by quantitative Western blotting (A) and its quantification (B). Data shown as mean ± SEM. *$P < 0.01$ *versus* vehicle; †$P < 0.01$ *versus* AII; #$P < 0.01$ *versus* AII + Val.

genic cytokines in the present study in the rat is similar to that recently reported in studies of the human kidney (32). The present findings provide evidence that in the kidney, there is an axis between angiotensin II and two major pathways implicated in proliferation, the VEGF–VEGF receptor and angiopoietin–Tie-2 receptor axes.

Previous studies by Yuan *et al.* (33) have characterized in detail the expression of the angiopoietins and Tie receptors in renal development. In the present study, the angiopoietins were also localized to the glomerular epithelial cells, collecting ducts, and vasa recta. Tie-2 receptor expression, as reported by several groups, was restricted to endothelial cells in the kidney (34). This included endothelial cells within glomeruli, cortical blood vessels, and vasa recta in the medulla (35–37). Although there were significant increases in Ang-1, Ang-2, and Tie-2 receptor expression in response to angiotensin II, there was no evidence of a change in distribution of these proteins within the kidney.

There is evidence that the growth stimulatory and profibrogenic effects of angiotensin II may not be solely transduced through the AT_1 receptor (38). Consistent with previous studies by our group (24), blockade of the AT_1 receptor with valsartan reduced the number of glomerular proliferating cells to control levels in the present study. It is interesting that blockade of the AT_2 receptor with PD123319 also demonstrated a significant reduction in glomerular proliferating cells, although to a lesser degree than valsartan. The proliferative action of the AT_2 receptor has been previously demonstrated *in vitro* in mouse spleen lymphocytes (39) and *in vivo* in mesenteric vessels (40). It is important to note that these antiproliferative effects of the AT_2 receptor are independent of the reduction of systemic BP.

It has been demonstrated that VEGF is produced in response to stretch in rat ventricular myocardium (41), rat cardiac myocytes (42), and human mesangial cells (43). Therefore, the attenuation of VEGF by valsartan treatment may be a direct effect of its BP-lowering ability. By contrast, the effects of the AT_2 receptor antagonist PD123319 are not associated with a reduction in BP. This emphasizes the nonhemodynamic effects of angiotensin II on VEGF and its receptor, VEGF-R2.

The finding that VEGF was increased by angiotensin II infusion *in vivo* is consistent with *in vitro* studies. For example, it has been shown that VEGF expression is stimulated *in vitro* by angiotensin II in both cardiac endothelial cells (12) and retinal pericytes (3). VEGF receptor expression in association with cellular proliferation has also been described in response to angiotensin II in retinal endothelial cells (13). With respect to the kidney, human mesangial cells have also been reported to increase VEGF expression in response to angiotensin II (44). In most of these *in vitro* studies, these effects are considered to have been mediated by the AT_1 receptor subtype. However, in the present study, which has been performed in an *in vivo* context, both AT_1 and AT_2 receptors seem to participate in mediating angiotensin II–induced changes in VEGF and VEGF-R2 expression.

Angiotensin II has also been shown previously to induce the expression of the angiopoietins *in vitro*, in particular Ang-2 (4,12). These *in vitro* studies were performed in retinal endothelial cells and must be interpreted with caution when extrapolating these findings to an *in vivo* context such as in this study, particularly in another organ, such as the kidney. Over the past few years, it has been considered that Ang-1 is the activator of the Tie-2 receptor, whereas Ang-2 is an endogenous antagonist (9). Initially, it was viewed that Ang-1 was more likely to be responsible for promoting VEGF-induced angiogenesis (9). However, there is increasing evidence that Ang-2, the natural antagonist of Tie-2, is a proangiogenic ligand (10,11,45). Recent studies, particularly using a cardiac expression system, have explored whether Ang-2 can act as a proangiogenic factor particularly in the context of a milieu with increased VEGF (10). In the present study, in which exogenous angiotensin II was administered, there was an increase in both angiopoietins in the context of an associated increase in VEGF. This combination of increased VEGF expression in association with increased angiopoietins led to glomerular proliferation, as one would predict on the basis of the recent *in vitro* studies. The concomitant increase in VEGF-R2 and Tie-2 would potentially amplify these proliferative effects induced by angiotensin II.

In the present study, AT_2 receptor blockade, although reducing gene expression of both angiopoietins, was not associated with decreased protein expression of either Ang-1 or Ang-2. It remains to be determined whether this disparity in effects on gene and protein expression are due to posttranscriptional regulatory mechanisms. However, minimal information is available about this issue with respect to expression of the

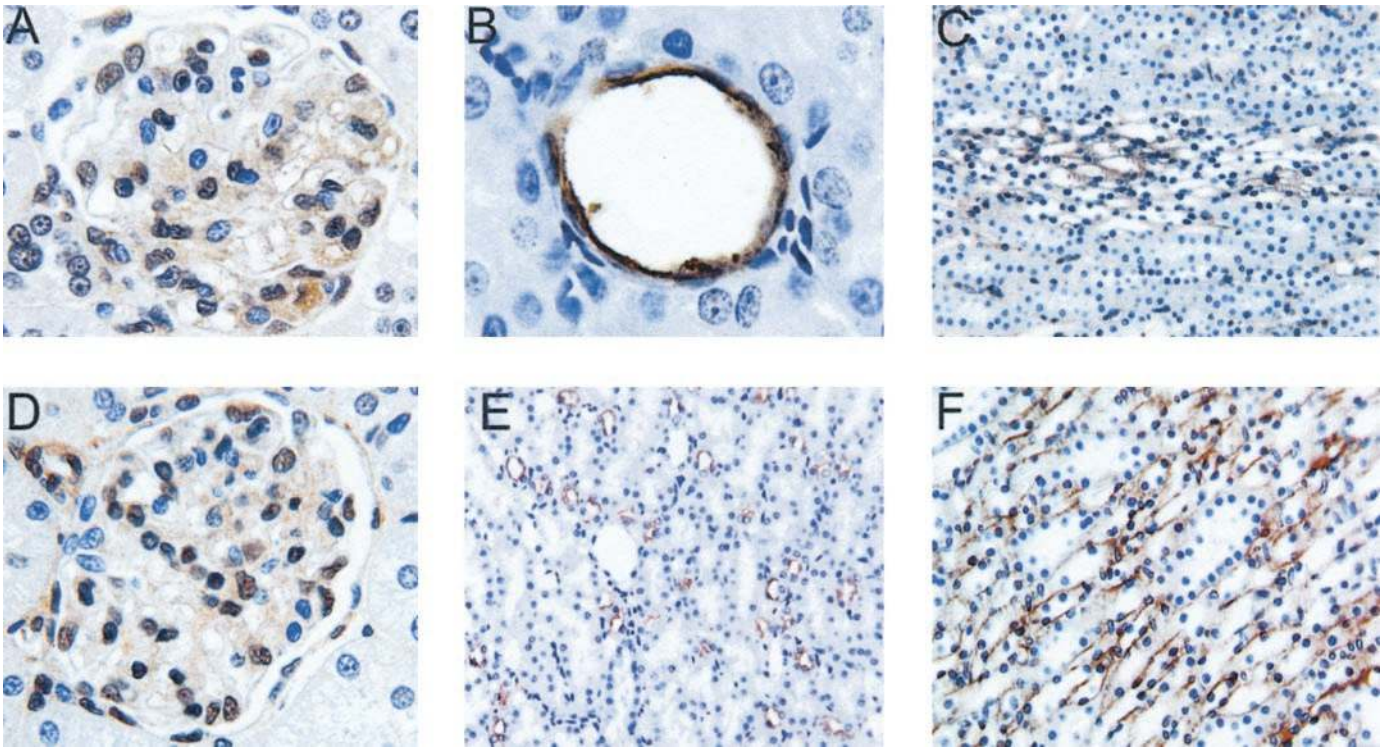

Figure 7. Immunohistochemical detection of Ang-1 (A to C) and Ang-2 (D to F) in epithelial cells of the glomerulus (A and D), collecting ducts (B and E), and vascular bundles of the medulla (C and F). Magnifications: ×400 in A, D, and F; ×1000 in B; ×200 in C and E.

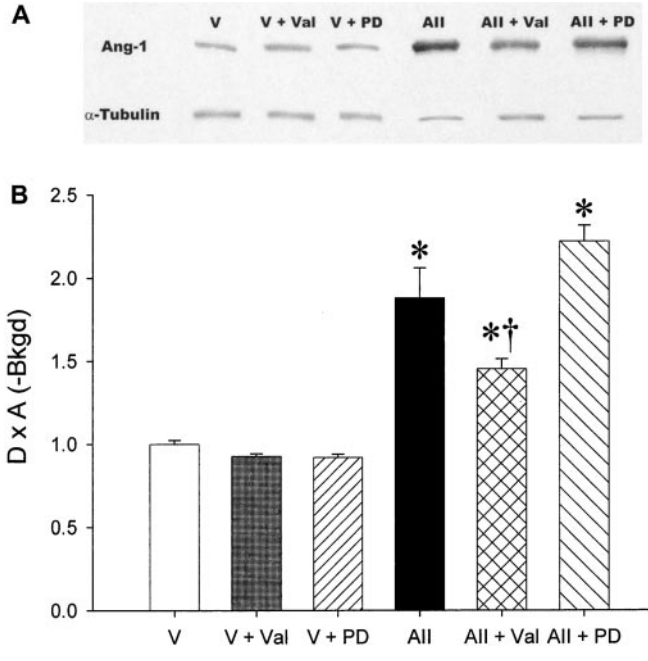

Figure 8. Photograph of a stained nitrocellulose membrane for Ang-1 protein by quantitative Western blotting (A) and its quantification (B). Data are shown as mean ± SEM. *$P < 0.01$ *versus* vehicle; †$P < 0.01$ *versus* AII.

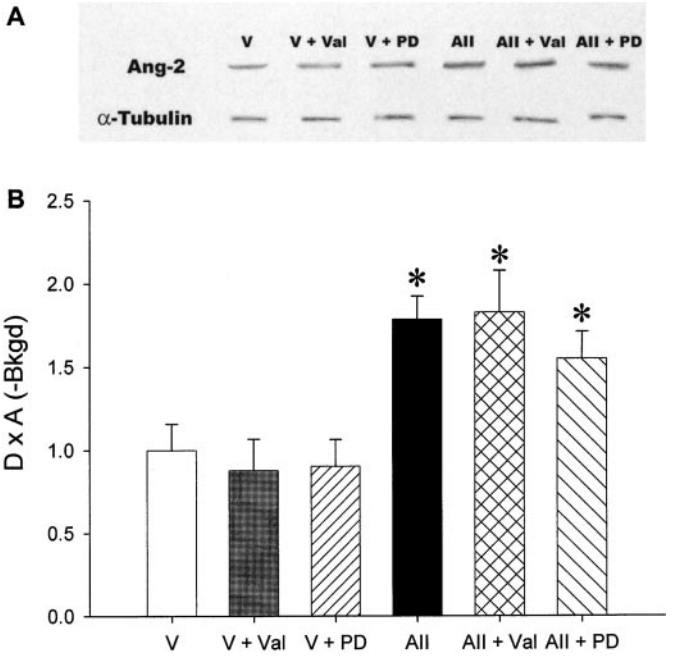

Figure 9. Photograph of a stained nitrocellulose membrane for Ang-2 protein by quantitative Western blotting (A) and its quantification (B). Data are shown as mean ± SEM. *$P < 0.01$ *versus* vehicle.

angiopoietins. By contrast, VEGF gene and protein expression was attenuated by both AT_1 and AT_2 receptor blockade in association with reduced glomerular proliferation, suggesting that VEGF-dependent pathways predominate in this setting. This is consistent with recent studies, emphasizing the predominance of VEGF over the angiopoietin pathways, performed in

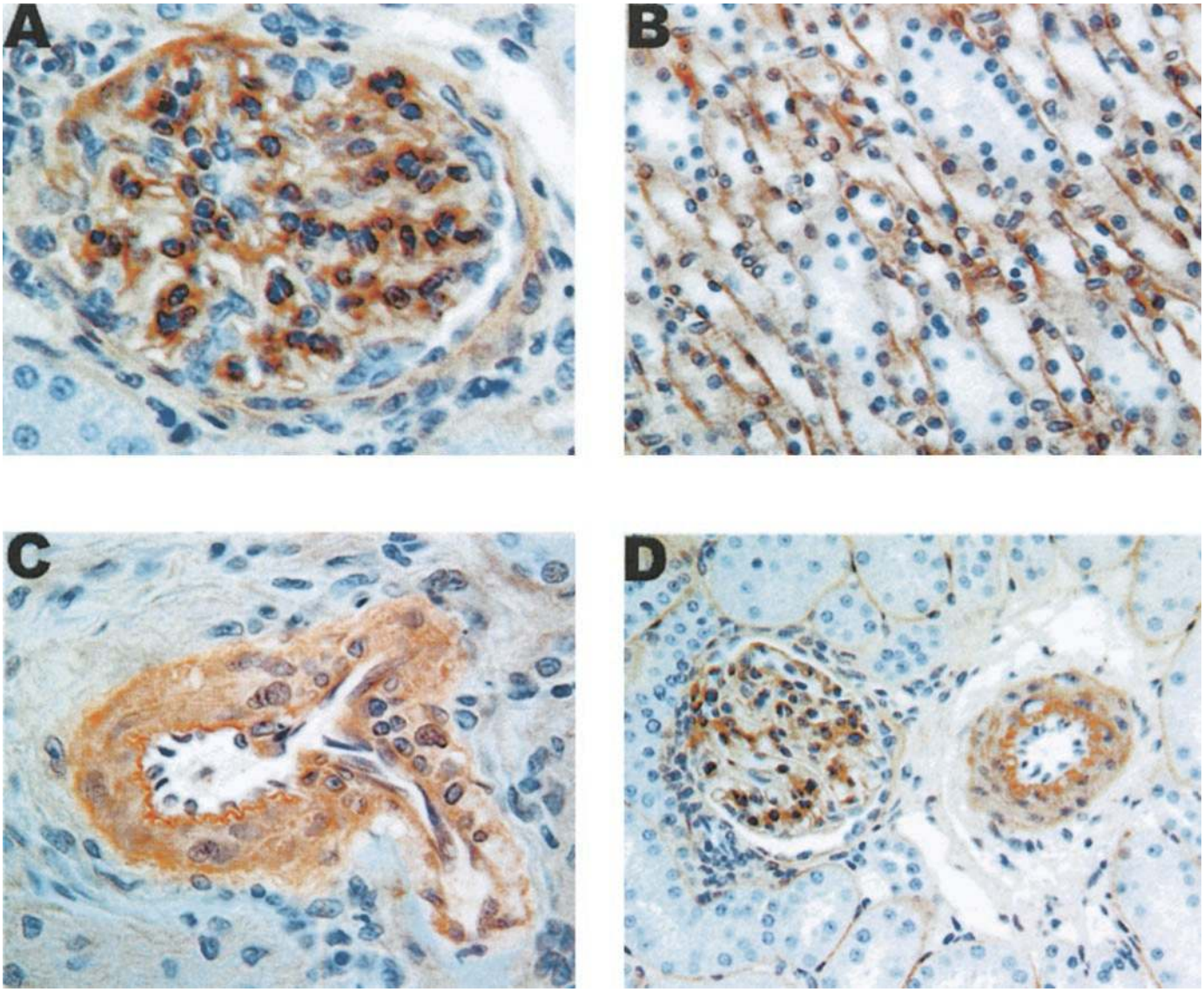

Figure 10. Immunohistochemical detection of Tie-2 receptor in endothelial cells of the glomerulus (A), vascular bundles of the medulla (B), and the endothelium of renal blood vessels of the cortex (C) of vehicle-infused rats. VEGF-R2 was also located in the endothelial cells of the glomerulus and cortical blood vessels (D). Magnifications: ×400 in A to C, ×200 in D.

other contexts such as in the ovary, where reduced VEGF expression resulted in vascular regression despite increased Ang-2 expression (46).

Over the past few years, an increased number of effects of the AT_2 receptor within the kidney have been described. For example, angiotensin II-induced expression of the chemokine RANTES is primarily AT_2 receptor dependent (47). Other effects of angiotensin II seem to be mediated by both receptor subtypes. Angiotensin II activates the nuclear transcription factor NF-κB via either the AT_1 or the AT_2 receptor (38). *In vivo* effects of angiotensin II on cellular proliferation and apoptosis seem to be mediated by both receptors. In a recent study of animals subjected to subtotal nephrectomy, AT_1 and AT_2 receptor blockade attenuated renal injury and proteinuria in association with effects on expression of the slit pore protein nephrin as well as reduced cellular proliferation (27). The present study extends these findings to effects on expression of VEGF and the angiopoietins providing evidence that both angiotensin II receptor subtypes participate in modulating expression of these proteins, which are clearly implicated in cellular proliferation and angiogenesis.

Administration of either AT_1 or AT_2 receptor antagonists in vehicle-infused rats had no significant influence on gene or protein expression of these angiogenic growth factors or their receptors. This indicates that in pathologic states such as angiotensin II infusion, both AT_1 and AT_2 receptors play a role in mediating the expression of these proangiogenic growth factors. Indeed, it is likely that the effects of angiotensin II on glomerular cellular proliferation occur via both angiotensin II receptor subtypes and involve both VEGF and angiopoietin pathways.

The present study has identified that angiotensin II is a major

stimulus of angiogenic cytokines and their receptors. Specifically, both AT_1 and AT_2 receptor blockade antagonize the actions of angiotensin II on the expression of these angiogenic cytokines VEGF, Ang-1, and Ang-2. It seems likely that these effects lead to changes in glomerular cellular proliferation, which could potentially play a role in the progression of renal disorders.

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