Kidney

**Regulation of Angiogenic Factors in Angiotensin II Infusion Model in Association With Tubulointerstitial Injuries**

Hiroyuki Kitayama, Yohei Maeshima, Yuki Takazawa, Yoshihiko Yamamoto, Yan Wu, Kunihiro Ichinose, Kumiko Hirokoshi, Hitoshi Sugiyama, Yasushi Yamasaki, and Hirofumi Makino

**Background:** Among various angiogenic factors, vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang1), and angiopoietin-2 (Ang2) play crucial roles in regulating angiogenesis and vascular integrity. Infusion of angiotensin-II (ang II) induces hypertension and focal renal tubulointerstitial injuries. In the present study we investigated the renal expression of VEGF, Ang1, Ang2, and corresponding receptors in association with tubulointerstitial lesions in a rat ang II infusion model.

**Methods:** Male Sprague-Dawley (SD) rats received an infusion of ang II or norepinephrine (NE) through osmotic minipumps for 14 days. Angiotensin II type 1 (AT1) or type 2 (AT2) receptor antagonist (losartan or PD123319, respectively) or hydralazine was co-administered.

**Results:** Interstitial fibrosis, infiltration of monocyte/macrophage, and peritubular capillary rarefaction induced by ang II was significantly attenuated in the losartan- or PD123319-treated groups. Immunoreactivity of VEGF and Ang1 in cortical tubules was increased by ang II and was attenuated by losartan or PD123319. The increase of VEGF induced by ang II was suppressed by losartan, and the increase of Ang1 induced by ang II was inhibited by PD123319 as detected by immunoblot. The increase of flk-1 and flt-1 (VEGF receptors) and tie-2 (Ang1 receptor) induced by ang II was significantly suppressed by PD123319. These alterations were not observed in hydralazine plus ang II or NE-infused animals.

**Conclusions:** These results demonstrate that an infusion of ang II induced the expression of VEGF mainly through AT1 receptors, and increased the expression of VEGF receptors, tie-2, and Ang1/Ang2 ratio mainly through AT2 receptors. The increase of VEGF/flk-1/flt-1 may be associated with vascular permeability, monocyte/macrophage infiltration, and rarefaction of peritubular capillaries, and the increase of the Ang1/Ang2 ratio may be a compensatory mechanism countering the permeability inducing effect of VEGF after ang II infusion. Am J Hypertens 2006;19:718–727 © 2006 American Journal of Hypertension, Ltd.

**Key Words:** Angiotensin II, interstitial fibrosis, angiogenesis, VEGF, angiopoietin-1, angiopoietin-2.

Progressive renal disorders are accompanied by the development of tubulointerstitial alterations characterized by interstitial infiltration of mononuclear cells, proliferation of interstitial fibroblasts, and accumulation of matrix proteins, leading to fibrosis. Deterioration of renal function correlates better with the degree of tubulointerstitial injuries rather than that of glomerular changes in many forms of glomerular disorders.

There are two major receptor isoforms of angiotensin II (ang II), AT1 receptor (AT1R) and AT2 receptor (AT2R).

The AT1Rs are expressed in arterioles, glomeruli, and proximal tubules in normal kidney, dominantly mediating the...
known biological functions of ang II. Angiotensin II induces renal injuries by vasoconstriction, growth promoting, as well as a profibrotic effect leading to excess accumulation of extracellular matrix (ECM).

Critical involvement of AT1R and possible renoprotective effect of AT2R in progressive renal disorders had been reported. Continuous infusion of ang II results in focal tubular atrophy, interstitial infiltration of monocyte/macrophage, interstitial fibrosis, and vascular sclerosis, accompanied by a reduction of peritubular capillary endothelial cells.

Angiogenesis, the development of new blood vessels from pre-existing ones, is involved in physiologic as well as pathologic conditions. Vascular endothelial growth factor (VEGF) promotes endothelial cell proliferation, migration, and tube formation. The essential role of VEGF in inducing vascular permeability in association with inflammation and edema had been reported. Two major receptors for VEGF are known as flt-1 and flk-1/KDR, and the latter mainly mediates a proangiogenic effect of VEGF. Angiopoietin-1 (Ang1) binds to receptor tie-2 and induces stable pericyte attachment leading to vascular maturation. Angiopoietin-2 (Ang2) competitively inhibits the binding of Ang1 to tie-2, and renders blood vessels to be “unstable.” In cooperation with VEGF, Ang1 promotes vascular network maturation, whereas Ang2 initiates neovascularization. In developing kidney, Ang1, Ang2, and tie-2 play pivotal roles in the maturation of glomeruli and blood vessels. Alterations in the level of Ang1 and Ang2 had been demonstrated in glomerulonephritis and folic acid-induced nephropathy models. More recently, up-regulation of VEGF, Ang1, Ang2, flk-1 and tie-2 in kidney had been demonstrated in experimental diabetic nephropathy.

The involvement of ang II in mediating an angiogenic response had been reported. Altered expression of VEGF, Ang1, and Ang2 after infusion of ang II was associated with proliferation of glomerular cells. In the anti-Thy-1 nephritis model, we observed that ang II infusion accelerated the recovery of glomerular endothelial cells, possibly by inducing VEGF, Ang1, flk-1, and tie-2. In the present study, we hypothesized that infusion of ang II may modulate the expression of angiogenesis-associated factors and receptors, possibly leading to altered vascular permeability in association with tubulointerstitial lesions. In addition, we investigated the involvement of AT1R- and AT2R-mediated signals in this regulatory mechanism using receptor selective antagonists.

Methods

Experimental Protocol

The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University Graduate School of Medicine and Dentistry. Studies were performed in male Sprague-Dawley (SD) rats (CLEA Japan, Tokyo, Japan). The animals had free access to tap water and standard rat chow. Rats weighing 200 g received continuous infusion of phosphate-buffered saline (PBS), ang II (200 ng/ml; Sigma, St. Louis, MO), or norepinephrine (NE, 600 ng/ml; Sigma) through subcutaneous osmotic minipumps (Alzet model 2002; Alza Corp., Palo Alto, CA). Rats were evaluated 14 days after the implantation of osmotic minipumps.

The following groups of animals were studied: 1) normotensive control with PBS infusion; 2) ang II infusion; 3) ang II infusion plus losartan (30 mg/kg/d by the drinking water; Merck, NJ); 4) ang II infusion plus PD123319 (7.9 mg/kg/d osmotic minipump; Sigma); 5) NE infusion; n = 5 for each group. The dosage of ang II, PD123319, and NE was determined according to previous publication by Wenzel et al and similar experiments from our laboratory. The dosage of losartan was determined according to the data sheet provided by DuPont Merck to achieve chronic 24-h blockade of ang II. No rats have died during the experimental period.

Blood and Urine Examination

Blood urea nitrogen (BUN), serum, urinary creatinine, sodium, and urinary total protein levels were measured by SRL, Inc. (Okayama, Japan), as previously described. Serum and urinary creatinine levels were measured by the enzymatic colorimetric method as described. Urinary total protein concentration was measured by standard Pyrogallol red method using MicroTP-AR (Wako Pure Chemical, Osaka, Japan). The creatinine clearance (Ccr) was calculated and expressed as milliliters per minute.

Systolic and Diastolic Blood Pressure

Arterial blood pressure was measured before the implantation of the osmotic minipumps and sacrifice using a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) by the tail–cuff method, as described previously.

Immunoblot

Immunoblot was performed as previously described. Briefly, the cortex of the kidneys were homogenized in radioimmunoprecipitation assay (RIPA) buffer at 4°C. After centrifugation at 14,000 rpm for 30 min at 4°C, the supernatant was collected and stored at −80°C until use. Samples (100 μg per each lane) were processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then the proteins were electrotransferred onto nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, Piscataway, NJ) followed by blocking and overnight incubation with rabbit anti-angiopoietin-1 (1:1000; Alpha Diagnostics, San Antonio, TX), goat anti-angiopoietin-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-VEGF (1:1000; Santa Cruz), rabbit anti-flk-1 (1:100; Santa Cruz), rabbit anti-flt-1 (1:1000; Santa Cruz), or rabbit anti-tie-2 (1:1000; Santa Cruz) antibodies at 4°C. After incubation with horse radish peroxide (HRP)-labeled-secondary antibodies for 1 h, signals were detected with ECL system (Amersham). Membranes were reprobed with rabbit anti-
actin antibodies (Bio-Rad, Hercules, CA) to serve as controls for equal loading. The density of each band was determined by using National Institutes of Health (NIH) image software, and expressed as a value relative to the density of the corresponding band obtained from actin immunoblot.

**Histologic Analysis**

At 14 days after the implantation of the minipumps, the kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin. Sections (3 μm thick) were stained with Masson trichrome for light microscopic observation. Interstitial fibrosis was quantitated as follows: color images were obtained as TIF files and then analyzed using Lumina Vision software (Mitani, Fukui, Japan). In each kidney more than 20 randomly selected fields (renal cortex) were examined under ×200 magnification and averaged. The interstitial fibrosis index in each field was expressed as fibrotic area (blue) in the interstitium relative to the total area. Histologic assessment was performed by the investigators in a blinded fashion.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described. For immunohistochemistry of ED-1, frozen kidney sections were used. For immunohistochemistry of VEGF, Ang1, and Ang2 (Santa Cruz), formalin-fixed paraffin-embedded sections (3 μm) were used. Sections were incubated with monoclonal anti-rat ED-1 (Cymbus Bio-technology, Hants, UK), rabbit anti-VEGF (Santa Cruz), and biotinylated anti-Ang1 (Alpha Diagnostics), and goat anti-Ang2 (Santa Cruz). A level of P < .05 was considered statistically significant.

**Statistical Analysis**

All values are expressed as mean ± SE. A Kruskal-Wallis test with post-hoc multiple comparisons and the Scheffe’s test were used for intergroup comparisons of multiple variables. Statistical analysis was performed with StatView software (Abacus Concepts, Berkeley, CA). A level of P < .05 was considered statistically significant.

**Results**

**Changes in Blood Pressure**

Infusion of Ang II or NE resulted in significantly elevated arterial blood pressure (BP) as compared to PBS. There was no significant difference in BP between ang

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**Table 1.** Blood pressure (BP) and parameters of renal function

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic BP (mm Hg)</th>
<th>Diastolic BP (mm Hg)</th>
<th>S-Cr (mg/dL)</th>
<th>DU-TP (mg)</th>
<th>S-Na (mEq/L)</th>
<th>DU-Na (mEq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>120 ± 12</td>
<td>79 ± 3</td>
<td>0.23 ± 0.01</td>
<td>17.0 ± 0.3</td>
<td>145 ± 1</td>
<td>1.38 ± 0.16</td>
</tr>
<tr>
<td>Ang II</td>
<td>163 ± 8*</td>
<td>105 ± 26*</td>
<td>0.37 ± 0.02*</td>
<td>17.6 ± 0.9</td>
<td>144 ± 1</td>
<td>1.64 ± 0.30</td>
</tr>
<tr>
<td>Ang II + Losartan</td>
<td>115 ± 13†</td>
<td>82 ± 10</td>
<td>0.29 ± 0.03</td>
<td>10.7 ± 0.5*</td>
<td>145 ± 1</td>
<td>1.65 ± 0.36</td>
</tr>
<tr>
<td>Ang II + PD123319</td>
<td>191 ± 11*</td>
<td>102 ± 12*</td>
<td>0.32 ± 0.05</td>
<td>12.5 ± 1.7*</td>
<td>146 ± 1</td>
<td>1.46 ± 0.68</td>
</tr>
<tr>
<td>Ang II + Hydralazine</td>
<td>107 ± 8†</td>
<td>68 ± 10</td>
<td>0.39 ± 0.10*</td>
<td>13.3 ± 1.4*</td>
<td>143 ± 2</td>
<td>1.84 ± 0.32</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>167 ± 22*</td>
<td>107 ± 6*</td>
<td>0.27 ± 0.01</td>
<td>17.0 ± 1.8</td>
<td>144 ± 1</td>
<td>1.53 ± 0.07</td>
</tr>
</tbody>
</table>

Ang II – angiotensin II; DU-Na – daily urinary sodium excretion; DU-TP – daily urinary protein excretion; S-Cr – serum creatinine levels; S-Na – serum sodium levels. Values are shown as mean ± SEM. n = 5 in each group.

* P < .05 v PBS; † P < .05 v Ang II.
Renal Function and Proteinuria

On day 14, significant renal dysfunction was observed in ang II-infused animals. Improvement of renal function was observed in the losartan-treated group without reaching statistical significance, but not in the PD123319- or hydralazine-treated group. The ang II infusion did not affect urinary protein excretion, and treatment with losartan or PD123319 lead to significantly reduced urinary protein levels as compared to untreated ang II-infused animals. Treatment with hydralazine also mildly decreased urinary protein levels as compared to the untreated ang II-infused group without reaching statistical significance. Infusion of NE affected neither renal function nor proteinuria. Although serum levels of sodium were not different among experimental groups, urinary sodium excretion was mildly increased in the ang II-infused groups without reaching statistical significance. Treatment with losartan, PD123319, or hydralazine did not significantly affect urinary sodium excretion in ang II-infused animals. Infusion of NE resulted in a mild increase of urinary sodium excretion compared to control animals receiving PBS infusion without reaching statistical significance (Table 1).

Histology and Morphometric Analyses

Focal interstitial fibrosis accompanied by tubular atrophy and thickened vessel walls was observed in the ang II-infused group. In contrast, tubulointerstitial alterations in NE-infused rats were less remarkable. Treatment with either losartan or PD123319 in addition to ang II infusion markedly diminished tubulointerstitial injuries. Treatment with hydralazine plus ang II infusion resulted in slightly diminished interstitial fibrosis as compared to ang II alone (Fig. 1 and Table 2).

Interstitial Infiltration of Monocyte/Macrophage

The number of monocyte/macrophage in the cortical interstitial area was significantly increased in the ang II-infused and ang II plus hydralazine-treated rats compared to PBS- or NE-infused rats (PBS control; 1.3 ± 0.3, NE infusion: 1.1 ± 0.2, ang II infusion; 4.1 ± 0.5, ang II plus hydralazine: 3.8 ± 0.4; Fig. 1 and Table 2). Accumulation of monocyte/macrophage induced by ang II was significantly suppressed in either the losartan- or PD123319-treated groups (losartan; 2.7 ± 0.5, PD123319; 2.5 ± 0.4).

RECA-1(+) Peritubular Capillary Area

We next examined the changes in peritubular capillary (PTC) density by immunohistochemistry with anti-RECA-1 antibody, a specific rat endothelial cell marker. The PTC area was significantly decreased in ang II-infused animals compared to control animals receiving PBS infusion, which is consistent with a previous report showing similar results. The NE infusion failed to show such an effect. Treatment with losartan and PD123319 resulted in significant recovery of PTC area compared to the untreated ang II-infused group.
Treatment with hydralazine failed to show such an effect on ang II-induced reduction of PTC area (Table 2 and Fig. 1).

**Immunohistochemical Analysis of VEGF**

In the renal cortex of PBS-infused rats, the VEGF protein was detected mildly in distal tubular epithelial cells and less markedly in proximal tubular epithelial cells. The ang II infusion resulted in a marked increase of VEGF in distal tubules and a moderate increase in proximal tubules compared to PBS- or NE-infused control rats. The increase of VEGF induced by ang II was partially suppressed by losartan or PD123319, but not by hydralazine (Fig. 2 and Table 3). In addition, intense immunoreactivity for VEGF was observed in the medulla, conceivably in the loop of Henle according to morphologic appearances, in ang II-infused animals (data not shown). The specificity of observed immunoreactivity for VEGF in ang II-infused animals was demonstrated by experiments in the presence of blocking peptide, exhibiting absent immunoreactivity (Fig. 2). The expression of Ang2 in PBS-infused rats was faintly observed in both proximal and distal tubules, and was not significantly altered by ang II infusion. Treatment with losartan, PD123319, or hydralazine did not exhibit significant alterations in the expression of Ang2 (Fig. 2 and Table 3). The expression of Ang2 in interlobular arteries and afferent/efferent arterioles was mildly observed in PBS-infused rats, and was increased in ang II-infused animals.

**Expression of VEGF and Receptors (immunoblot)**

The expression of VEGF, flk-1, and flt-1 in the renal cortex was examined by immunoblot. The level of VEGF was significantly elevated in ang II-infused rats (3.2-fold) compared with PBS, but not in NE-infused animals. The ang II-induced increase of VEGF was potently suppressed by losartan (69.5% inhibition), and mildly by PD123319 (27.6% inhibition) (Fig. 3A,B). The expression of flk-1 was significantly increased in ang II-infused rats (1.8-fold) compared to the PBS- or NE-infused group (Fig. 3E,F). Angiotensin II-induced increase of flk-1 was weakly suppressed by losartan (26.1% inhibition), and markedly suppressed by PD123319 (125% inhibition) (Fig. 3E,F). The expression of flt-1 was significantly increased in ang II-infused rats (1.7-fold) compared to the PBS- or NE-infused group (Fig. 3C,D). The ang II-induced increase of flt-1 was weakly suppressed by losartan (28.7% inhibition), and markedly suppressed by PD123319 (128% inhibition) (Fig. 3C,D). Treatment with hydralazine failed to affect the induction of VEGF, flk-1, or flt-1 after ang II infusion.

**Expression of Ang1, Ang2, and tie-2**

The expression of Ang1, Ang2, and tie-2 was further examined by immunoblot. The level of Ang1 was significantly elevated by ang II infusion (2.8-fold) compared to PBS, but not in NE-infused animals. Although the ang II-induced increase of Ang1 was not affected by losartan, PD123319 treatment resulted in marked suppression (77.8% inhibition) (Fig. 4A,B). Treatment with hydral-
shown (original magnification, \( \times 100 \)). Note absent immunoreactivity in the presence of blocking peptide (\( \times 200 \)).

**FIG. 2.** Immunohistochemistry of VEGF, Ang1, and Ang2 (renal cortex). Indirect immunohistochemistry was performed as described in the Methods section. Immunohistochemistry of VEGF for PBS infused (A), ang II infused (B), losartan plus ang II infused (C), PD123319 plus ang II infused (D), hydralazine plus ang II infused (E), and norepinephrine infused (F) groups (arrowheads – distal tubules with intense immunoreactivity for VEGF). In ang II-infused rats, VEGF expression was observed intensely in distal tubules and mildly in proximal tubules. Increased immunoreactivity for VEGF induced by ang II infusion was attenuated in losartan- or PD123319-treated groups. Immunohistochemistry of Ang1 for PBS infused (G), ang II infused (H), losartan plus ang II infused (I), PD123319 plus ang II infused (J), hydralazine plus ang II infused (K), and norepinephrine infused (L) groups (arrowheads – distal tubules with intense immunoreactivity for Ang1). In ang II-infused rats, Ang1 expression was observed intensely in distal tubules and mildly in proximal tubules. Expression of Ang1 induced by ang II infusion was attenuated in PD123319-treated groups. Immunohistochemistry of Ang2 for PBS infused (M), ang II infused (N), losartan plus ang II infused (O), PD123319 plus ang II infused (P), hydralazine plus ang II infused (Q), and norepinephrine infused (R) groups. (S and T) Primary antibodies were preincubated with blocking peptide and processed for immunostaining using sections obtained from kidneys of ang II-infused rats. Anti-VEGF antibody plus blocking peptide (S) and anti-Ang1 antibody plus blocking peptide (T). Note absent immunoreactivity in the presence of blocking peptide, indicating the specific immunoreactivity for VEGF and Ang1. Representative light microscopic appearance of renal cortex is shown (original magnification, \( \times 200 \)).

**Discussion**

The involvement of ang II in the process of renal tubulo-interstitial injury has been largely demonstrated to date. Angiotensin II promotes the phenotypic alteration of fibroblasts to myofibroblasts, resulting in interstitial deposition of ECM. The role of ang II in inducing profibrotic-transforming growth factor-\( \beta1 \) has been reported. Angiotensin II is also involved in the inflammatory process through the synthesis of chemotactic factors such as monocyte chemoattractant protein-1 (MCP-1), osteopontin, and RANTES. The involvement of ang II in regulating angiogenesis in various setting of diseases had been described previously. Angiotensin II stimulated the expression of VEGF and Ang2, and induced angiogenesis through AT1R in cardiac microvascular endothelial cells. In contrast, the possible action of AT2R in inducing angiogenesis was reported using AT2R-deficient mice. In the diabetic retinopathy model and the ang II infusion model, increased retinal expression of VEGF was mediated by AT1R and AT2R.

Significant deterioration of renal function was observed in ang II-infused animals, and was mildly improved by losartan but not evidently by PD123319 or hydralazine. A previous report demonstrated that infusion of ang II for a shorter term (7 days) in SD rats decreased glomerular filtration rate (GFR; as demonstrated by Ccr) and increased proteinuria. They described that the AT1R-mediated action of ang II was responsible for decreased renal function, whereas the synergistic action of ang II and pressor overload was critical for the increased proteinuria. Increased serum creatinine levels in ang II-infused animals compared to control rats are in line with this previous observation. In the present study, ang II infusion did not increase urinary protein excretion, and treatment with losartan or PD123319 led to significantly reduced urinary protein levels compared to untreated ang II-infused animals. The discrepancy between our findings and previous findings regarding proteinuria may be attributed to differences in time points and the extent of hypertension exhibiting milder hypertension in the present study compared to a previous report. Treatment with hydralazine also mildly decreased urinary protein levels compared to the untreated ang II-infused group, consistent with previous findings. Infusion of
NE affected neither renal function nor proteinuria, suggesting a pressor-independent action of ang II on renal dysfunction.

In the present study, focal interstitial infiltration of monocyte/macrophage and interstitial fibrosis was induced by ang II in contrast to NE, suggesting that the specific effect of ang II is independent of systemic hemodynamics. A previous report by Aizawa et al demonstrated that ang II infusion for 7 days resulted in increased renal tubular and interstitial cell apoptosis in contrast to NE infusion. Our findings showing diminished tubulointerstitial alterations by NE infusion in contrast to ang II-infused animals are consistent with these results. Therapeutic effects of losartan observed in the present study are consistent with

### Table 3. Immunostaining score for Ang1, Ang2, and VEGF

<table>
<thead>
<tr>
<th>Protein</th>
<th>PBS</th>
<th>Ang II</th>
<th>Losartan</th>
<th>PD123319</th>
<th>Hydralazine</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>0.83 ± 0.09</td>
<td>2.04 ± 0.13*</td>
<td>1.32 ± 0.10*†</td>
<td>1.54 ± 0.10*§</td>
<td>1.95 ± 0.14*</td>
<td>0.95 ± 0.09†</td>
</tr>
<tr>
<td>Ang1</td>
<td>1.21 ± 0.10</td>
<td>2.15 ± 0.09*</td>
<td>1.89 ± 0.12*†</td>
<td>1.64 ± 0.15†‡</td>
<td>2.15 ± 0.17*§</td>
<td>0.95 ± 0.09‡</td>
</tr>
<tr>
<td>Ang2</td>
<td>1.15 ± 0.13</td>
<td>1.20 ± 0.13</td>
<td>1.30 ± 0.11</td>
<td>1.20 ± 0.09</td>
<td>1.16 ± 0.09</td>
<td>1.20 ± 0.16</td>
</tr>
</tbody>
</table>

The grading score of immunoreactivity for VEGF, Ang1, and Ang2 in tubulointerstitium is shown. Values are shown as mean ± SEM. 

*P < .001 v PBS; †P < .03 v PBS; ‡P < .001 v Ang II; §P < .05 v Ang II.

![FIG. 3. Immunoblot analysis of VEGF, flk-1, and flt-1. In each lane, 100 μg of protein obtained from kidney cortex was loaded. Each band was scanned and subjected to densitometry. (A) Immunoblots for VEGF (30 kD) and actin (42 kD) are shown. (B) Intensities of VEGF protein relative to actin are shown. *P < .01 v C; †P < .01 v Ang II; ‡P < .05 v PD or H. (C) Immunoblots for flk-1 (170 kD) and actin (42 kD) are shown. (D) Intensities of flk-1 protein relative to actin are shown. *P < .01 v C; †P < .01 v Ang II; ‡P < .03 v L or H. (E) Immunoblots for flt-1 (160 kD) and actin (42 kD) are shown. (F) Intensities of flt-1 protein relative to actin are shown. *P < .01 v C; †P < .01 v Ang II; ‡P < .03 v L or H. C = PBS-infused control; A-II = Ang II infused; L = losartan plus ang II infused; PD = PD123319 plus ang II infused; H = hydralazine plus ang II infused; NE = norepinephrine infused. n = 5 for each group. Each column consists of mean ± SEM.](image)

![FIG. 4. Immunoblot analysis of Ang1, Ang2, and tie-2. In each lane, 100 μg of protein obtained from kidney cortex was loaded. Each band was scanned and subjected to densitometry. (A) Immunoblots for Ang1 (100 kD, most likely presenting as dimer) and actin (42 kD) are shown. (B) Intensities of Ang1 protein relative to actin are shown. *P < .01 v C; †P < .01 v Ang II; ‡P < .05 v L or H. (C) Immunoblots for Ang2 (50 kD) and actin (42 kD) are shown. (D) Intensities of Ang2 protein relative to actin are shown. *P < .01 v C; †P < .05 v Ang II; ‡P < .03 v Ang II. (E) Immunoblots for tie-2 (140 kD) and actin (42 kD) are shown. (F) Intensities of tie-2 protein relative to actin are shown. *P < .01 v C; †P < .01 v Ang II; ‡P < .05 v L or H. C = PBS-infused control; A-II = Ang II infused; L = losartan plus ang II infused; PD = PD123319 plus ang II infused; H = hydralazine plus ang II infused; NE = norepinephrine infused. n = 5 for each group. Each column consists of mean ± SEM.](image)
known renoprotective effects of AT1R blockade. A previous report demonstrated remarkable therapeutic effects by losartan in a similar model in reversing tubulointerstitial and vascular injuries, suggesting the dosage of losartan used in the present study to be insufficient to completely block these histologic alterations. Treatment with the nonspecific vasodilator hydralazine failed to show significant inhibitory effects on ang II-induced tubulointerstitial alterations, suggesting the therapeutic effect of losartan mediated through a pressor independent mechanism. Although the inhibitory effect on interstitial fibrosis was less remarkable compared with losartan, PD123319 exhibited an inhibitory effect on monocyte/macrophage infiltration comparable to losartan in spite of persistent hypertension. In fact, PD123319-treated ang II-infused animals exhibited slightly increased BP compared to untreated ang II-infused animals, possibly through enhanced AT1R-mediated signal accompanied by AT2R blockade.

A previous report described that transient exposure to pressor doses of ang II resulted in microvascular injury with PTC loss. In the present study, we examined the density of PTC in experimental groups by immunofluorescent staining using RECA-1 antibody. Similar to previously reported results as described above, the PTC area was significantly decreased in ang II-infused animals compared to control animals receiving PBS infusion. However, infusion of NE failed to show such an effect, suggesting the pressor-independent effect of ang II on PTC rarefaction. Treatment with losartan and PD123319 resulted in significant recovery of PTC area compared to the untreated ang II-infused group, suggesting the involvement of both AT1R and AT2R in this mechanism. Treatment with hydralazine failed to show such effect on ang II-induced reduction of the PTC area, further suggesting a pressor independent action of losartan.

We then investigated the possible involvement of VEGF and angiopoietins in ang II-induced focal tubulointerstitial alterations.

The increase of VEGF in the renal cortex induced by ang II was significantly inhibited by losartan, but only mildly by PD123319, suggesting the dominant involvement of AT1R. Although treatment with hydralazine resulted in decreased BP equivalent to losartan-treated animals, the increase of VEGF induced by ang II was not affected. On the other hand, NE infusion exhibiting hypertension comparable to ang II infusion failed to increase the renal expression of VEGF. These findings indicate that elevated BP was not responsible for up-regulation of VEGF, but rather this effect was attributable to the specific action of ang II. Immunohistochemical analysis demonstrated increased immunoreactivity for VEGF induced by ang II infusion mainly by AT1R in parallel with findings from immunoblots. Recent study demonstrated that ang II infusion up to 2 weeks resulted in increased expression of VEGF protein, and that AT1RA (valsartan) moderately suppressed VEGF production, in contrast to PD123319, which almost completely blocked ang II-induced increase of VEGF. The reason for this discrepancy may be attributable to the difference in the kidney samples. We obtained cell lysates from renal cortex and not from whole kidney materials. Different regulation of VEGF in response to ang II in distinct nephron segments might have led to this discrepancy. In addition, mean systolic BP of ang II-infused animals was 49 mm Hg lower, and AT1RA treatment also resulted in 24 mm Hg lower systolic BP compared with the report by Rizkalla et al. These differences might account for the discrepancy between the two studies. However, our results on AT1R-induced up-regulation of VEGF are consistent with a previous report on cardiac endothelial cells. Considering the known role of VEGF in promoting vascular permeability, these results suggest the possible involvement of VEGF in developing inflammatory lesions after ang II infusion. On the other hand, chronic ang II infusion induces renal vasoconstriction leading to renal ischemia. Because transcriptional activation of VEGF is partly mediated by hypoxia through the increase in the level of hypoxia-inducible factor-1, the ang II-induced increase of VEGF may reflect hypoxic conditions in the kidney due to renal ischemia. Next, we examined the level of VEGF receptors, flk-1 and flt-1. Flk-1 is mainly expressed on endothelial cells and involved in VEGF-induced mitogenesis, angiogenesis, and vascular permeability. Flt-1 is expressed on endothelial cells as well as monocyte/macrophages and induces angiogenesis as well as chemotaxis of monocytes. The protein level of flk-1 and flt-1 was increased by ang II infusion, mainly mediated by AT2R. Collectively, with chronic infusion of ang II, AT1R mediated up-regulation of VEGF and AT2R mediated up-regulation of VEGF mildly and of flk-1 and flt-1, the latter possibly accounting for the observed therapeutic effects of PD123319. Because VEGF is known to induce the expression of connective tissue growth factor (CTGF), which induces excess accumulation of extracellular matrix, ang II-induced increase of interstitial fibrosis may be associated with an up-regulation of VEGF/flk-1/flt-1.

We next examined the expression of angiopoietins. The level of Ang1 was increased by ang II mainly through AT2R in contrast to a previous report by Rizkalla et al, demonstrating the involvement of AT1R. The expression of Ang2 was mildly increased by ang II infusion, partially mediated through AT2R. Although the extent of the increase of Ang2 by ang II infusion was milder in comparison with a previous report, the suppressive effect by PD123319 was similarly observed. The expression of tie-2 was markedly increased by ang II through AT2R in parallel with Ang1. These results implicate that ang II infusion increased the relative ratio of Ang1/Ang2 and the expression of tie-2, and thus triggering the signal mediated by the Ang1/tie-2 system. This regulatory mechanism on the expression of Ang1/Ang2/tie-2 was mediated mainly through AT2R.

The appropriate balance between VEGF/flk-1/flt-1 and
Ang1/Ang2/tie-2 is important for proper formation and maintenance of blood vessels. Thurston et al. reported the formation of leaky blood vessels in mice overexpressing VEGF, and nonleaky vessels in mice overexpressing Ang1. We previously reported the increase of VEGF and Ang2 in a diabetic nephropathy model that was inhibited by antiangiogenic tu-mastatin peptide accompanied by therapeutic effects. In the present study, the VEGF protein was observed mainly in tubular epithelium, suggesting the possible permeability inducing action of VEGF acting on adjacent peritubular capillary endothelial cells, implicating an alternative mechanism of ang II-mediated inflammatory response in addition to known mechanisms including stimulation of NF-κB and chemokines. Therapeutic efficacy of Ang1 associated with anti-inflammatory bioactivities has been reported in diabetic retinopathy. In this context, increase of the Ang1-to-Ang2 ratio and tie-2 induced by ang II might be a compensatory mechanism counteracting the effect of VEGF in inducing vascular permeability. Presumably VEGF signaling might be excessive compared with required Ang1/tie-2 signaling in this model, thus leading to increased monocyte/macrophage infiltration. However, the potential involvement of angiogenesis-associated factors in this model need to be further validated by strategies such as specific overexpression or deletion of VEGF or Ang1. In the present study, infusion of ang II resulted in PTC rarefaction, which was blocked by losartan or PD123319. We speculate that the expression of VEGF and Ang1 by tubules adjacent to injured peritubular capillaries may be enhanced in ang II-infused animals as a compensatory mechanism to normalize PTC density. Because VEGF and Ang1 were mainly observed in tubular compartments, these angiogenic factors may exert angiogenic effects on peritubular capillaries by a paracrine manner. Reduction in the levels of VEGF, Ang1, and receptors upon treatment with these ang II receptor antagonists may be partly explained by increased PTC area with less necessity for inducing angiogenic stimuli.

Treatment with ang II results in an increased production of aldosterone, which has been shown to induce neovascularization by up-regulating the expression of angiogenic factors such as VEGF. Although our results on the unaffected levels of serum and urinary sodium in experimental groups do not clearly indicate the induction of aldosterone, local induction of aldosterone by ang II infusion might be involved in the induction of angiogenic factors.

In conclusion, chronic ang II infusion resulted in the induction of VEGF, Ang1, and corresponding receptors in association with focal tubulointerstitial alterations. Our findings may add a distinct mechanism involving angiogenesis-associated factors known to have beneficial effects on the RAS blockade in renal disorders. Further clarification of downstream signaling pathways involved in the regulation of VEGF, Ang1, Ang2, and flk-1/flt-1/tie-2 receptors mediated by AT1R or AT2R may lead to the development of novel therapeutic approaches for regulating tubulointerstitial injuries associated with various renal disorders.

References


