Angiotensin II formation in the kidney and nephrosclerosis in Ren-2 hypertensive rats

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Abstract

Background. Ren-2 transgenic hypertensive rats develop malignant hypertensive nephrosclerosis despite low to normal plasma angiotensin II and suppressed renal renin. We tested the hypothesis that local angiotensin II formation occurs at sites of renal vascular and interstitial injury in this model.

Methods. Heterozygous Ren-2 transgenic rats were compared with normotensive Sprague–Dawley–Hannover control rats and Ren-2 transgenic rats treated with a very low dose of an angiotensin II type 1 (AT₁) receptor antagonist, 1mg/kg/day losartan, for 4 weeks. Blood pressure measurements, quantifications of urinary albumin, plasma and tissue angiotensin II as well as immunohistochemical analyses were performed.

Results. Systolic blood pressure was not affected by losartan during the study but intra-arterial recordings revealed a decrease of blood pressure. Losartan reduced albumin excretion, cell proliferation, macrophage influx, collagen I and collagen IV deposition. Plasma angiotensin II was decreased, while kidney tissue angiotensin II content was increased in Ren-2 transgenic rats compared with control rats. In Ren-2 transgenic rats, juxtaglomerular renin and angiotensin II staining were reduced, but there was a marked angiotensin II staining at foci of tubulo-interstitial fibrosis and at proliferative malignant vascular lesions.

Conclusion. We conclude that local angiotensin II formation is increased in proliferative or fibrotic kidney lesions in the Ren-2 transgenic rat. Local angiotensin II formation may help to explain why the AT₁ receptor antagonist prevents or ameliorates this transgenic model of malignant nephrosclerosis despite low to normal plasma angiotensin II and suppressed renal renin.

Keywords: angiotensin II; AT₁ receptor antagonist; hypertension; nephrosclerosis; Ren-2 transgenic rats

Introduction

Hypertensive nephrosclerosis is an important complication of arterial hypertension. However, the mechanisms which lead to hypertensive nephrosclerosis are not completely understood. Other factors in addition to blood pressure might contribute to progressive alterations in the kidney. Angiotensin II is an effective mediator of cell proliferation and matrix synthesis [1–3]. Some authors argued that activation of the renin–angiotensin system seems to be involved in chronic interstitial fibrosis in some forms of hypertension [4]. Inhibition of angiotensin II formation by ACE inhibitors or blockade of the angiotensin II type 1 receptor (AT₁) reduce renal injury, even if blood pressure is not substantially lowered [5]. In contrast, others [6,7] have argued that the apparent non-haemodynamic effects of angiotensin II blockade may in fact be due to blood pressure lowering. Many clinical trials published during the last 5 years found that the potential of AT₁ receptor antagonists or ACE inhibitors to protect the kidney from hypertensive injury was superior in comparison with other anti-hypertensive drugs [8–11]. One very large trial, however, did not confirm this notion [12]. Nevertheless, the widely acknowledged renoprotective effects of angiotensin II antagonists have given rise to the hypothesis that a local intrarenal renin–angiotensin system could contribute to renal injury. During the last decade, several authors have described high levels of angiotensin II in kidney tissue or in...
Local angiotensin II and nephrosclerosis

Measurement of plasma angiotensin II

Plasma angiotensin II was measured by direct radioimmunoassay with an antibody 100% cross-reactive with angiotensin III and IV, as described before [28,29].

Measurement of tissue angiotensin II

Renal tissue angiotensin II concentration was determined by a modification of the method described by Kai et al. [16]. Briefly, collected kidneys were snap frozen in liquid nitrogen and boiled in a 10-fold volume (w/v) of 0.05 N HCl solution for 10 min. Kidneys were homogenized with a Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany) and the debris removed by centrifugation at 12,000 g at 4°C for 1 h. Before HPLC purification, the supernatant was applied to a bond-elut pH column (Varian, Darmstadt, Germany) which had been pretreated consecutively with 8 ml methanol, 5 ml of the mixture of methanol/water/trifluoroacetic acid (TFA) (10/89.9/0.1 vol%) and 5 ml TFA 0.1%. After sample application, the column was washed with 5 ml TFA 0.1% and 8 ml of methanol/water/TFA (10/89.9/0.1 vol%). Peptides were eluted from the cartridge with 2 ml of the mixture of methanol/water/TFA (80/19.9/0.1 vol%) and concentrated to a volume of approximately 200 μl in a vacuum centrifuge evaporator. To the eluate, 700 μl of 0.01 M ammonium acetate buffer, pH 5.4 was added and the sample chromatographed on a Nucleosil-C18 reverse phase high-performance liquid chromatography (HPLC) column (Machery & Nagel, Düren, Germany) at 42°C. The separation of angiotensin II was effected by using a linear gradient of methanol concentration from 35 to 80% in 0.01 M ammonium acetate, pH 5.4 over a period of 25 min at a flow rate of 1.0 ml/min. Fractions of 0.5 ml were collected into bovine serum albumin (BSA)—coated polypropylene tubes and completely dried in a vacuum centrifuge evaporator. The fractionated samples were dissolved in radioimmunoassay buffer (0.1 M Tris-acetate buffer, pH 7.4) and subjected to a radioimmunoassay specific for angiotensin II (see aforementioned).

This method described by Kai et al. [16] is very similar to a protocol extensively used by Navar and Nishiyama [18] to determine kidney tissue angiotensin II content. We determined the HPLC elution time of angiotensin II by UV detection of the effluent after the addition of high amounts of peptides, as described previously [28]. To validate the measurements, a small amount of radioactively labelled angiotensin II was added to each individual sample before extraction. This radioactivity did not disturb the radioimmunoassay because the elution time of iodinated angiotensin II is different from that of the native peptide. The total recovery after extraction and HPLC ranged from 68 to 78%, and was not different between groups. Peptide levels were not corrected for recovery.

Antibodies and immunohistochemistry

Rabbit polyclonal antibodies to renin and angiotensin II were applied as described before [29,30]. A second antibody to angiotensin II (Bachem, Heidelberg, Germany) was applied at a dilution of 1:200 to confirm the results obtained from immunohistochemical studies with the first antibody. Sections from the previously described stroke-prone,
spontaneously hypertensive rats (SHR-SP) [31] and deoxycorticosterone-acetate (DOCA) salt hypertensive animals [32] were also stained for angiotensin II. Rabbit polyclonal antibodies to the matrix proteins collagen I (Biogenesis, Poole, England) and collagen IV (Southern Biotechnology Associates, Birmingham, AL, USA) were used at a dilution of 1:1000. A mouse monoclonal antibody detecting proliferating cells (PCNA) was purchased from Santa Cruz Biotechnologies (Heidelberg, Germany) and used at a dilution of 1:50. The mouse monoclonal antibodies to macrophages (ED-1) and activated macrophages (ED-3) were from Serotec (Biozol, Eching, Germany) and diluted 1:250. The mouse monoclonal antibody to alpha-smooth-muscle actin was from Serotec (Biozol, Eching, Germany).

Immunohistochemistry was performed in deparaffinized sections of methyl-Carnoy fixed kidneys, using an avidin horseradish peroxidase detection system (Vector Lab, Burlingame, CA) as described before [32]. For angiotensin II and ED-1 double-immunostaining, sections were stained for ED-1 first, followed by blocking the peroxidase activity and subsequent staining for angiotensin II. To detect angiotensin II staining, the Vector VIP substrate kit for peroxidase (Vectastain) was used, resulting in a purple staining. Stained sections were embedded in Entellan (Merck, Darmstadt, Germany). As a negative control, we used equimolar concentrations of pre-immune rabbit or mouse immunoglobulin G, or an irrelevant rabbit primary antibody. Double-immunostainings for angiotensin II and smooth-muscle actin were detected by immunofluorescence: Primary antibodies were applied simultaneously overnight at 4°C. After washing, sections were incubated with secondary antibodies, CY2 labelled goat anti-mouse immunoglobulin G and CY3 labelled goat anti-rabbit immunoglobulin G, both from Dianova (Hamburg, Germany), at the same time for 2 h. Washed sections were then covered with Tris-buffered Mowiol, pH 8.6 (Hoechst, Frankfurt, Germany).

Quantification of immunohistochemistry

Interstitial PCNA, ED-1 or ED-3 positive cells were counted in 20 medium-power (magnification 250×) cortical views per section and expressed as cells per square millimetre. Intraglomerular PCNA, ED-1 or ED-3 positive cells were counted in all the glomeruli of a given section (150–300) and expressed as cells per glomerular cross-section. Counting of angiotensin II- and renin-positive juxtaglomerular apparatus was performed as described before [30].

To evaluate tubulo-interstitial collagen I or collagen IV, computer-based integration of stained areas was performed in 10 low-power views per kidney section (Metaview, Visitron Systems, Puchheim, Germany). The average area staining positive for collagen I or collagen IV was calculated as a percentage of total cortical area.

Analysis of data

Two-way analysis of variance, followed by post-hoc Newman–Keuls test, was used to test significance of differences between groups. A P-value <0.05 was considered significant. The procedures were carried out using the SPSS software (release 9.01, SPSS Inc., Chicago, IL, US). Values are displayed as means±SEM.

Results

In Ren-2 transgenic hypertensive rats, systolic and mean arterial blood pressure was markedly increased (Table 1, Figure 1). Relative kidney and heart weights, creatinine clearance, protein excretion (Table 1) as well as albumin

### Table 1. Organ weights, plasma angiotensin II (Ang II), angiotensin II- and renin-positive juxtaglomerular apparatus, creatinine clearance, proteinuria and mean arterial blood pressure measurements

<table>
<thead>
<tr>
<th></th>
<th>Kidney weight/ body weight ratio (mg/g)</th>
<th>Heart weight/ body weight ratio (mg/g)</th>
<th>Plasma Ang II (fmol/ml)</th>
<th>Ang II-positive JGA (%)</th>
<th>Renin-positive JGA (%)</th>
<th>Creatinine clearance (ml/min)</th>
<th>Proteinuria (mg/mg creatinine)</th>
<th>Mean arterial pressure (mmHg)</th>
</tr>
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<tbody>
<tr>
<td>SD</td>
<td>3.22±0.09</td>
<td>3.33±0.11</td>
<td>73±8</td>
<td>16.0±3.0</td>
<td>15.6±4.6</td>
<td>7.34±0.95</td>
<td>3.46±0.18</td>
<td>120.1±6.5</td>
</tr>
<tr>
<td>TGR</td>
<td>4.21±0.18*</td>
<td>5.23±0.27*</td>
<td>58±7*</td>
<td>7.9±2.9*</td>
<td>8.6±2.7*</td>
<td>2.07±0.69*</td>
<td>23.07±6.53*</td>
<td>189.2±8.8*</td>
</tr>
<tr>
<td>TGR + AT1RA</td>
<td>3.60±0.12*</td>
<td>4.37±0.2*</td>
<td>–</td>
<td>–</td>
<td>4.73±1.6*</td>
<td>6.09±0.94*</td>
<td>10.5±0.88*</td>
<td>171.6±10.4*</td>
</tr>
</tbody>
</table>

SD, Sprague–Dawley–Hannover control rats; TGR, Ren-2 transgenic hypertensive rats; AT1RA, Angiotensin II type 1 receptor antagonist; Ang II, angiotensin II; JGA, juxtaglomerular apparatus. Data are means±SEM, *P<0.05 vs SD, †P<0.05 vs TGR.
excretion (Figure 1) were significantly higher in Ren-2 transgenic hypertensive rats as compared with Sprague–Dawley–Hannover controls. Plasma angiotensin II of Ren-2 transgenic hypertensive rats was decreased compared with Sprague–Dawley–Hannover controls (Table 1). Also, juxtaglomerular angiotensin II and renin staining were clearly reduced in Ren-2 transgenic hypertensive rats (Table 1). Treatment with a low dose of an AT1 receptor antagonist did not affect systolic blood pressure (Figure 1), while mean arterial pressure measurements at the end of the treatment period revealed a small but significant decrease in AT1 receptor antagonist-treated Ren-2 transgenic hypertensive rats (Table 1). The relative weights of heart and kidney, as well as creatinine clearance, proteinuria and albuminuria were markedly reduced in AT1 receptor antagonist-treated Ren-2 transgenic hypertensive rats (Table 1).

Fig. 2. Infiltration of total macrophages (A and B) and activated macrophages (C and D) into the renal interstitium (A and C) or the glomerulus (B and D) of experimental groups. SD, Sprague–Dawley–Hannover control rats; TGR, Ren-2 transgenic hypertensive rats; AT1RA, angiotensin II type 1 receptor antagonist. Data are means±SEM, *P<0.05 vs SD, §P<0.05 vs TGR. (E) Representative examples of ED-1 immunohistochemistry in renal sections of SD, TGR and TGR + AT1RA.

An increase in interstitial as well as glomerular matrix expansion was detected in Ren-2 transgenic hypertensive rats compared with Sprague–Dawley–Hannover controls (Figure 3C). Treatment with AT1 receptor antagonist reduced interstitial collagen I expansion significantly (Figure 3C and D), while glomerular collagen IV expansion was only marginally decreased (4.3±0.7% of glomerular area in Ren-2 transgenic hypertensive rats treated with AT1 receptor antagonist vs 4.9±0.4 in Ren-2 transgenic hypertensive rats and 2.1±0.3 in Sprague–Dawley–Hannover controls, ns).

In Sprague–Dawley–Hannover controls, angiotensin II immunoreactivity was confined to the juxtaglomerular apparatus (Figure 4A). In contrast, renal tissue of Ren-2 transgenic hypertensive rats displayed angiotensin II immunoreactivity also in areas of tubulo-interstitial injury (Figure 4B) and surrounding proliferative malignant vascular lesions (Figure 4C, D and F), which was never seen in Sprague–Dawley–Hannover controls (data not shown) or after staining with pre-immune serum (Figure 4E). Surprisingly, this staining could not be completely blocked with excess exogenous angiotensin II (data not shown). However, use of another angiotensin II antiserum, and of
different staining methods (acetone-fixed cryosections instead of paraffin-embedded, methyl-Carnoy fixed tissue; fluorescence-labelled secondary antibody for detection) invariably showed positive angiotensin II immunostaining in areas of tubulo-interstitial and vascular injury (Figure 4D). Double staining for angiotensin II and smooth muscle actin revealed a partial colocalization (Figure 4G). In contrast, double staining with the macrophage marker ED-1 showed no localization of angiotensin II immunoreactivity within ED-1 positive cells but angiotensin II staining surrounding macrophages (Figure 4H).

This kind of angiotensin II staining was not seen in losartan-treated rats, and mostly absent in kidney sections from rats with other forms of hypertension: Some angiotensin II immunostaining was rarely encountered in SHR-SP rat kidneys surrounding malignant vascular lesions (Figure 4I). The DOCA-salt hypertensive rats displayed occasional angiotensin II staining in tubules but not in interstitial or vascular areas (data not shown) despite prominent renal damage [32].

Finally, HPLC analysis of kidney extracts revealed that immunoreactive angiotensin II was mostly due to the authentic octapeptide. In all samples analysed, more than 85% of total angiotensin II immunoreactivity eluted in the angiotensin II peak. Moreover, kidney tissue angiotensin II levels were elevated in TGR (Figure 5).

Discussion

Despite low plasma angiotensin II and suppressed juxtaglomerular renin and angiotensin II levels, Ren-2 transgenic hypertensive rats developed malignant hypertensive nephrosclerosis which could be significantly ameliorated by administration of a low dose of AT1 receptor antagonist. The drug did lower blood pressure from around 190 mmHg to the still very high level of about 170 mmHg. Heart and kidney hypertrophy, albuminuria, cell proliferation, macrophage infiltration and interstitial matrix expansion were markedly reduced or normalized after treatment with an AT1 receptor antagonist. Angiotensin II was detected at sites of interstitial fibrosis and proliferative malignant vascular lesions in Ren-2 transgenic hypertensive rats. Inhibition of the activity of the locally generated angiotensin II may contribute to the beneficial effects of AT1 receptor blockade in this model of angiotensin II-dependent hypertensive nephrosclerosis.

The existence of locally active renin–angiotensin systems has been described before [33]. In the kidney, components of the renin–angiotensin system were described in tubular epithelial cells [34–36], and tubular angiotensin II staining increased in response to subtotal nephrectomy, [15]. Others have described high angiotensin II levels in tubular fluid [14,17,37]. These findings suggest that tubular angiotensin II might contribute to tubulo-interstitial injury. In contrast, we detected immunoreactive angiotensin II in the Ren-2 transgenic rat in areas of interstitial and vascular injury, not in tubules. In agreement with our findings, tubular fluid angiotensin II is not elevated in this model of hypertension [23]. The immunostaining experiments did not appear fully conclusive because we could not block the angiotensin II immunoreactivity completely by exogenous angiotensin peptides. While we are unable to explain this phenomenon, several lines of evidence indicate that the immunostaining of these areas represents ‘true’ angiotensin II. First, we could confirm the staining with another antiserum against angiotensin II whereas pre-immune serum caused no staining. Second, the staining was reproducible with several different methods of detection and tissue preparation. Third, HPLC measurements confirmed that angiotensin II was elevated in transgenic rat.
kidneys, and that the vast majority of immunoreactive angiotensin II represented the authentic octapeptide. Double staining with cell markers demonstrated that immunoreactive angiotensin II was partially localized to vascular smooth muscle cells (or activated myofibroblasts expressing smooth muscle actin), and was often present in a paracellular localization adjacent to macrophages. Whether the angiotensin II was generated by locally synthesized renin and angiotensinogen [15], or by uptake of these components from plasma [38], will require further study.

Our finding that intrarenal angiotensin II is elevated in Ren2-transgenic rats agrees with previous reports which suggested high levels of the peptide in the kidney [25,39], but is at variance with other investigations in heterozygous Ren2-transgenic rats [22–24].

![Fig. 4. Immunohistochemical detection of angiotensin II in paraffin-embedded kidney sections. (A) Reactive juxtaglomerular apparatus in SD. (B) Interstitial angiotensin II staining at the site of tubulo-interstitial injury in TGR. (C) Angiotensin II staining in a fibrotic vascular lesion in TGR. (D) Immunofluorescent detection of angiotensin II in frozen kidney sections by a commercially available angiotensin II antibody to confirm the results obtained in paraffin-embedded sections. (E and F) Specificity control of the antibody to angiotensin II: two consecutive sections were either treated with pre-immune rabbit serum (E) or with the angiotensin II antibody (F). (G) Double immunofluorescence for angiotensin II (red) and smooth-muscle actin (green). (H) Double immunohistochemistry for angiotensin II (purple) and ED-1 (brown). (I): Immunohistochemical detection of angiotensin II in renal vascular lesions of SHR-rats. Scale bars of 50 µm are included in most photomicrographs except for panel H because, the maximum width shown on this panel is 50 µm. SD, Sprague–Dawley–Hannover control rats; TGR, Ren-2 transgenic hypertensive rats.](#)

![Fig. 5. Kidney angiotensin II levels as determined by RIA and HPLC. SD, Sprague-Dawley–Hannover control rats; TGR, Ren-2 transgenic hypertensive rats. Data are means±SEM, *P<0.05 vs SD.](#)
Kopkan et al. [22] described decreased angiotensin II in kidney tissue whereas Mitchell et al. [23] and Senanayake et al. [24] found no difference to control animals. We can only speculate on the reasons for this discrepancy. Kopkan et al. [22] as well as Mitchell et al. [23] relied on immunoreactive angiotensin II measurement by radioimmunooassay while we used HPLC purification. The choice of control animals, age and gender of the rats, as well as the drug used for anesthesia, may play a role. Further, there is also some controversy regarding the status of plasma angiotensin II which is, at least in part, due to the difficulty to avoid in vitro activation of the very high plasma prorenin in these animals (for review, see Peters et al. [40]). However, plasma angiotensin II, if measured in samples drawn from indwelling catheters in conscious, male, heterozygous Ren-2 transgenic rats, is usually slightly suppressed if compared with suitable control animals [41], as reported here. Elevated plasma angiotensin II has been reported in different situations, for example in homozygous Ren-2 transgenic rats [25].

Despite normal or slightly decreased plasma angiotensin II, hypertension and target organ injury in heterozygous Ren-2 transgenic rats are known to be highly susceptible to blockers of the renin–angiotensin systems, even at low doses [39]. Local angiotensin II formation in several organs, including the adrenal gland [42] and the blood vessels [27], may account for this phenomenon. Our findings and previous reports of others point to the role of local angiotensin II formation in the kidney. In addition to its haemodynamic effects, angiotensin II exerts several actions which may contribute to the pathological changes observed in transgenic rat kidneys. The peptide has growth-promoting activity [3] and it is able to up-regulate matrix expression [43]. The contribution of angiotensin II to matrix expansion seems to be mediated by the cytokine TGF-β which is induced by angiotensin II [43]. Further, angiotensin II promotes inflammation via induction of the transcription factor NF-kB [44]. The role of these non-haemodynamic mechanisms relative to blood pressure effects is difficult to ascertain in vivo. We cannot exclude the possibility that the beneficial effects of AT1 blockade observed in our study are due to the antihypertensive effect. However, we point out that the blood pressure effect was small, and that the degree of amelioration of functional and structural kidney abnormalities in Ren-2 transgenic rats by AT1 blockade was remarkable for a treatment started in established hypertension.

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Conflict of interest statement. R.V and K.F.H. have received grant support and speaker’s fees from several pharmaceutical companies which manufacture AT1 receptor antagonists. There are no relationships to disclose for the remaining authors.

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