Angiotensin II Receptor Type 1 Gene Expression in Human Glomerulonephritis and Diabetes Mellitus

JÜRGEN WAGNER, FRANK GEHLEN, ANDRZEJ CIECHANOWICZ, and EBERHARD RITZ
Department of Nephrology, Medizinische Klinik, University of Heidelberg, Heidelberg, Germany.

Abstract. The renin-angiotensin system plays an important role in the progression of chronic renal disease. Although the expression of renin and angiotensin-converting enzyme in experimental and human renal disease has been well characterized, no information is available regarding human angiotensin type 1 (AT1) receptor expression. The net effect of renin depends on AT1 receptor expression, among other factors. Receptor expression was determined in renal biopsy samples (including all tissue components) and isolated glomeruli from patients with glomerulonephritis (GN) or diabetic nephropathy (non-insulin-dependent diabetes mellitus). Biopsy samples and isolated glomeruli from tumor-free tissue from tumor nephrectomies served as controls. Human AT1 receptor gene expression was determined by quantitative reverse transcription-PCR, using an AT1 receptor deletion mutant as the internal standard. In whole biopsy samples from 37 patients with various types of GN, AT1 receptor mRNA levels were lower, compared with nine control biopsy samples (P < 0.001). AT1 receptor mRNA levels were also significantly lower (P < 0.001) in eight samples from patients with diabetic nephropathy. In microdissected glomeruli, AT1 receptor gene expression was significantly lower in samples from patients (n = 22) with various types of GN, compared with 12 microdissected tumor nephrectomy control samples (P < 0.0023). It is concluded that AT1 receptor mRNA expression is low in glomeruli of patients with chronic renal disease. This may reflect a regulatory response to (inappropriately) high intrarenal angiotensin II concentrations.

Clinical and experimental studies have identified the renin-angiotensin system (RAS) as a key factor in the progression of renal failure. Pharmacologic blockade of this system, e.g., using angiotensin-converting enzyme (ACE) inhibitors or angiotensin II (AngII) type 1 (AT1) receptor antagonists, retards progression of glomerulosclerosis (1–5).

Although the effects of ACE inhibitors or AT1 receptor blockers on progression in renal patients have been studied extensively, renal expression and regulation of the components of the RAS have not been well investigated in human subjects. Such analysis is important, because both Nakamura et al. (6) and we (7) have documented that changes in the plasma RAS do not closely reflect local expression of the renal RAS. Complete assessment of intrarenal expression and regulation of all components of the RAS is necessary for evaluation of the net effect of the renin system on the kidney. In this context, expression of the AT1 receptor is of particular importance.

In normal human kidneys, the AT1 receptor is the main receptor for AngII in the glomerulus. It is also expressed in the brush border and basolateral membranes of the proximal tubules, in the vasculature, and in other components of the kidney (8). Experimental studies indicate that renal AT1 receptor binding is reduced in the kidneys of rats with streptozotocin-induced diabetes, as well as after administration of glucocorticoids (9,10). No information, however, is available regarding renal and glomerular AT1 receptor expression in the kidneys of patients with chronic renal disease. This prompted us to determine AT1 receptor gene expression in kidney biopsy specimens and in microdissected glomeruli of patients with a broad spectrum of chronic renal diseases.

Materials and Methods

Patient Data

Determination of AT1 Receptor mRNA Levels in Whole-Kidney Biopsy Samples. Controls for Whole-Kidney Biopsy Samples. Kidney samples were obtained from nine tumor nephrectomy specimens (samples from five women and four men; age range, 48 to 73 yr; median age, 63.5 yr; systolic BP, 135 ± 2.6 mmHg; diastolic BP, 81.6 ± 1.2 mmHg). Renal biopsies were obtained at the time of surgery from patients with renal cell carcinoma, from sites remote from tumor-bearing tissue. Patients had not undergone thrombembolization of the renal artery before surgery.

Glomerulonephritis. Thirty-seven patients (24 men and 13 women; median age, 37.5 yr; age range, 18 to 64 yr; systolic BP, 127 ± 2.6 mmHg; diastolic BP, 80 ± 1.2 mmHg) with glomerulonephritis (GN), i.e., IgA nephritis (n = 19), membranous glomerulopathy (n = 4), membranoproliferative GN (n = 2), minimal-change disease/focal segmental glomerulosclerosis (n = 6), or systemic vasculitis (n = 6), were examined; they exhibited 24-h proteinuria (2.8 ± 0.7 g, n = 35) and creatinine clearance rates of 78.4 ± 6.6 ml/min (n = 30). The patients had no dietary restrictions. None of the glomerulonephritic patients was receiving steroids, immunosuppressive drugs, or contraceptives. One patient was receiving β-blockers,
and two were receiving calcium antagonists. Nine of the patients were receiving ACE inhibitors and/or diuretic agents.

**Non-Insulin-Dependent Diabetes Mellitus.** Eight patients (five men and three women) with non-insulin-dependent diabetes mellitus (who were receiving oral antidiabetic treatment and exhibited overt proteinuria) underwent biopsies because the diagnosis was uncertain. All exhibited diabetic nephropathy (median age, 59 yr; age range, 39 to 84 yr; systolic BP, 141 ± 4.2 mmHg; diastolic BP, 85 ± 2.5 mmHg; creatinine clearance, 79.2 ± 18 ml/min; 24-h proteinuria, 2.9 ± 1.0 g/24 h). Four of the patients were receiving ACE inhibitors.

**Determination of AT₅ Receptor mRNA Levels in Isolated Glomeruli.** Controls for Microdissected Glomeruli. From 12 tumor nephrectomy specimens, both whole biopsy samples and isolated glomeruli were obtained at the time of surgery. Isolated glomeruli obtained from tumor-free tissue of tumor nephrectomy specimens served as controls (see Table 1 for clinical data). The control samples were analyzed at the same time as the glomerulonephritic samples.

**Glomerulonephritis.** Isolated glomeruli were recovered from individual biopsy specimens by microdissection (see Table 1 for clinical data). Diagnoses included IgA nephritis (n = 11), membranous glomerulopathy (n = 3), minimal-change disease/local segmental glomerulosclerosis (n = 6), and membranoproliferative GN (n = 2).

**Biopsy Technique**

Patients underwent biopsies for diagnostic purposes. One biopsy was obtained for diagnostic analysis and one for measurement of AT₁ receptor gene expression. Written informed consent was obtained from the patients before the biopsies. The study was approved by the local ethics committee. Biopsies were obtained from the left lower pole, under ultrasonographic guidance (Toshiba Sonolayer; Toshiba Medical Systems, Neuss, Germany), using a Biopty system (Radiplast AB, Uppsala, Sweden) and an 18-gauge (1.2-mm) needle, as described elsewhere (7).

To obtain control samples, renal biopsies were obtained immediately after ligation of the renal artery, with 10 min of warm ischemia time. A nephrologist was present at the operating table. The Biopty needle was directed perpendicularly to the surface so that sampling conditions were similar to those for standard renal biopsies. For determination of whole-biopsy sample mRNA expression, specimens were placed in a presterilized reaction tube (Eppendorf, Hamburg, Germany), shock-frozen in liquid nitrogen, and stored at −80°C until further analysis.

**Microdissection**

Microdissection of glomeruli and subsequent reverse transcription (RT) were performed essentially as described by Peten et al. (11). In brief, glomeruli were collected from biopsy samples at 4°C, in microdissection buffer containing 135 mM NaCl, 1 mM Na₂HPO₄, 1.2 mM Na₂SO₄, 1.2 mM MgSO₄, 5 mM KCl, 2 mM CaCl₂, 5 mM Hepes, and 5.5 mM glucose. Vanadyl ribonucleoside complex (10 mM; Life Technologies) served as RNAse inhibitor.

Microdissection was performed in 10-ml dishes on ice, in precooled microdissection buffer containing vanadyl ribonucleoside complex, under a stereomicroscope (Carl Zeiss, Jena, Germany). Five glomeruli were pooled in one tube (0.2-ml reaction tubes; Eppendorf) containing microdissection buffer, RNAsin (Promega), and 5 mM dithiothreitol, essentially as described (12).

**Quantification of AT₁ Receptor Gene Expression**

The Trizol (Life Technologies) method was used for RNA isolation from whole biopsy specimens, according to the manufacturer’s recommendations. Total RNA isolated from each biopsy sample ranged from 2.6 to 18 μg. Selected biopsy specimens were checked for degradation of total RNA using gel electrophoresis.

RNA concentrations were determined by spectrophotometric measurements at 260/280-nm wavelengths. Measurements were performed in triplicate.

**Amplification Method**

Total RNA from whole biopsy samples was reverse-transcribed to cDNA according to the method of Wang et al. (13). Total RNA (0.5 μg) was reverse-transcribed at 22°C for 10 min, 42°C for 45 min, and 94°C for 5 min, in 20 μl of a reaction mixture containing 1 mM levels of dATP, dCTP, dTTP, and dGTP, 40 U of RNAsin, 5 μM random hexamers (all reagents from Boehringer), 50 mM KCl, 20 mM Tris-

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**Table 1. Clinical data for individuals included in the study on microdissected glomeruli**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n = 12)</th>
<th>Glomerulonephritis (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>9/3</td>
<td>14/8</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>43 to 73</td>
<td>23 to 60</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>61 ± 9</td>
<td>42 ± 12b</td>
</tr>
<tr>
<td>ACE inhibitors/diuretics (n)</td>
<td>2/12</td>
<td>7/22</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>115 to 155</td>
<td>110 to 160</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>132 ± 12</td>
<td>128 ± 11</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>75 to 90</td>
<td>75 to 100</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>84 ± 5</td>
<td>83 ± 6</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
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<tr>
<td>range</td>
<td>0.7 to 1.3</td>
<td>0.9 to 2.8</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>1.1 ± 0.2</td>
<td>1.7 ± 0.6b</td>
</tr>
</tbody>
</table>

a ACE, angiotensin-converting enzyme.

b p < 0.05 versus control.
HCl (pH 8.4), 2.5 mM MgCl₂, 1 mg/ml nuclease-free bovine serum albumin, and 200 U of Superscript reverse transcriptase (all reagents from Life Technologies). For amplification of the resulting cDNA, the sample volume was increased to 100 μl with a solution containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 1 mg/ml nuclease-free bovine serum albumin, 0.8 mM nucleotide triphosphate mixture, 80 nM levels of sense and antisense primers (Biotez, Berlin, Germany), and 2.5 U of Taq polymerase (Life Technologies). The primers were 5'-TGTAAGATGCTTCAGCCAGC-3' (467 to 485 bp, sense primer) and 5'-GCGGTGTCCACAATATCTGC-3' (873 to 892 bp). For construction of the cDNA mutant, the amplification product was cloned into the pGEM-T-vector (Promega, Madison, WI). A Ball/Ball fragment was then removed by enzymatic cleavage and subsequently ligated to create a deletion mutant. The thermal profile used in a Perkin Elmer/Cetus thermal cycler consisted of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, for 30 cycles. After 30 cycles, the PCR was in the linear phase of the reaction (data not shown). Ten microliters of the amplification products was checked for products of the predicted size by agarose gel electrophoresis.

Quantitative RT-PCR of AT₁ Receptor mRNA

Quantification of specific mRNA was performed essentially as described (14), with the following modifications (15). Reverse-transcribed RNA (0.5 μg) was used for amplification in the presence of defined concentrations of DNA deletion mutants as internal standards. The concentration of standard DNA was selected to allow comparable degrees of amplification of endogenous and mutant genes, facilitating signal determination. Ten microliters of the amplification products was separated by agarose gel electrophoresis, digitized using a gel documentation system (Intas, Göttingen, Germany), and subjected to analysis using National Institutes of Health Image 1.44 software (15). The optical density values of the amplification products were determined in arbitrary units, and a ratio between the optical density of the endogenous cDNA and the optical density of the mutant DNA was obtained. Each sample was measured in triplicate individual PCR assays for each gene.

For quantification of glomerular AT₁ receptor mRNA, a different approach was used. After destruction of cell membranes with Triton X-100, RT was started in situ at 42°C. For each PCR, a solution containing the cDNA of one-tenth of a glomerulus from five pooled glomeruli was used. For each measurement, five PCR were performed, each containing the same amount of cDNA but increasing concentrations of internal standard (11,12). The PCR products were electrophoretically separated, and band intensities were determined by video scanning as described above (15). For determination of glomerular AT₁ receptor mRNA expression, we used 36 cycles.

Statistical Analyses

Data are expressed as mean ± SEM or median as appropriate. Data were analyzed using the nonparametric Mann–Whitney test. Statistical significance was accepted at P < 0.05.

Results

Quantitative RT-PCR of the AT₁ Receptor Gene

Figure 1A depicts the amplification signals of the endogenous gene and the mutant cDNA. Figure 1B gives an example of a dilution series from the PCR, as used for video scanning.

Figure 1. PCR of angiotensin II type 1 (AT₁) receptor mRNA. (A) Amplification products of endogenous (AT1R) and mutant (AT1RM) cDNA. CON, control. (B) Dilution series of a quantitative reverse transcription-PCR assay.

AT₁ Receptor mRNA in Whole-Kidney Biopsy Samples from Patients with Various Types of GN

Patients and control subjects were not significantly different with respect to systolic BP (control, 135 ± 2.6 mmHg; GN, 127 ± 2.6 mmHg), diastolic BP (control, 81.6 ± 1.5 mmHg; GN, 80 ± 1.2 mmHg), or S-creatinine levels (control, 1.046 ± 0.04 mg/dl; GN, 1.47 ± 0.15 mmHg) (Figure 2). The age of the control group was somewhat higher (62.1 ± 2.9 yr versus 40 ± 2.1 yr; P < 0.01).

AT₁ receptor expression was lower for glomerulonephritic patients (n = 37; 3.57 ± 0.56 arbitrary optical density units/μg of total RNA) compared with control subjects (n = 9; 20.8 ± 0.27 arbitrary optical density units/μg of total RNA; P < 0.001) in whole-kidney biopsy samples. No significant differences in AT₁ receptor gene expression were noted between patients: (1) with creatinine clearance rates of >60 or <60...
ml/min (although AT₁ receptor mRNA levels tended to be lower in advanced renal failure) (Figure 3A); (2) with more “inflammatory” renal diseases (IgA nephritis or vasculitis) compared with less inflammatory types of GN (membranous GN, minimal-change disease/focal segmental glomerulosclerosis) (Figure 3B); or (3) with or without ACE inhibition (P < 0.1) (Figure 3C).

AT₁ Receptor mRNA in Whole-Kidney Biopsy Samples from Patients with Non-Insulin-Dependent Diabetes Mellitus

AT₁ receptor message levels in whole-kidney biopsy samples were significantly lower in samples from patients with non-insulin-dependent diabetes mellitus than in control samples (P < 0.001), despite similar serum creatinine levels and systolic and diastolic BP values (Figure 4).

AT₁ Receptor mRNA in Isolated Glomeruli of Glomerulonephritic Patients

Compared with glomeruli isolated from 12 microdissected control tumor nephrectomy biopsy samples, AT₁ receptor mRNA levels were significantly lower in glomeruli from 22 patients with GN (P < 0.0023) (Figure 5; see Table 1 for clinical data).

Discussion

A salient finding of this study is the observation that AT₁ receptor gene expression is low in chronic renal disease. Whole biopsies are a composite of various renal structural components, and the AT₁ receptor is expressed not only in glomeruli but also in vascular, tubular, and interstitial tissue. Therefore, we also examined isolated glomeruli to obtain some information regarding the topography of AT₁ receptor mRNA expression. The observation of low AT₁ receptor mRNA expression was confirmed in isolated glomeruli.

Quantitative RT-PCR is a technique that is well suited for quantification of mRNA in specimens as small as renal biopsies or isolated glomeruli. The technique has been used to monitor the expression of genes in human renal biopsies, e.g., renin, transforming growth factor-β1, collagen, or other genes (7,11,16,17). Several aspects of this technique deserve comment. Different PCR assays and strategies have been developed to quantify gene expression and to account for the variability of amplification efficiencies in these assays (13,18–21). Internal standards, which are included in the reaction mixture, are most commonly used (21). We used a deletion mutant that is coamplified with the endogenous gene as an internal standard. Using the same primer sites for the mutant and for the endogenous gene ensures comparable amplification efficiencies if the size difference between the mutant and endogenous gene products does not exceed 30% (18,22,23). This requirement was met in our case. The specificity of the assay has been shown by restriction enzyme analysis of the amplification products. The linearity of the assay has been determined beyond 32 cycles for amplification from whole-kidney biopsy samples and beyond 38 cycles for isolated glomeruli. To enhance the reliability of the assay, we used dilution series of

Figure 3. AT₁ receptor (AT1R) mRNA expression in glomerulonephritic patients. (A) Comparison of the effect of creatinine clearance rates of <60 or >60 ml/min on AT₁ receptor expression. (B) Comparison of AT₁ receptor gene expression in noninflammatory and inflammatory renal disease. (C) Comparison of AT₁ receptor gene expression for patients with and without angiotensin-converting enzyme (ACE) inhibitor treatment.
each reverse-transcribed RNA amplified by PCR, to allow calculation from regression analysis instead of from single-point measurements, as performed previously (7,14,15). For the preparation of isolated glomeruli, we essentially followed the procedure described by Carome et al. (12), who added increasing amounts of deletion mutant to constant amounts of reverse-transcribed RNA. RT-PCR was repeated when the correlation coefficient was $<0.8$. With these safety measures, the coefficient of variation was $<20\%$. This is comparable to other quantitative measurements using RT-PCR or Northern blot analysis (13,14,24). To avoid RNA degradation, biopsy specimens were placed in reaction tubes immediately after renal biopsy and were instantly shock-frozen in liquid nitrogen. During this time period, no overt RNA degradation was detectable (7). For isolation of glomeruli, biopsy samples were placed in ice-cold buffer containing an RNAse inhibitor (12).

An interesting trend toward higher AT-1 receptor mRNA levels was noted for patients receiving treatment with ACE inhibitors. The difference was not statistically significant ($P > 0.1$), presumably because of the small sample size. Nevertheless, the observation is interesting, because it suggests that the technique was sensitive enough to detect changes in mRNA levels in a directionally appropriate manner. An increase in AT$_1$ mRNA levels after ACE inhibitor treatment was also found in vascular smooth muscle cells and whole kidneys of experimental animals (25,26). Interestingly, a trend toward lower AT$_1$ receptor mRNA levels was also seen in patients with more advanced renal failure, i.e., creatinine clearance rates of $<60$ ml/min. For ethical reasons, we could not obtain biopsies from nonanesthetized control patients. We considered the possibility that anesthesia might influence AT$_1$ receptor mRNA expression, but we regard this possibility as unlikely because of the short duration of anesthesia before biopsy. We also considered the possibility that replacement of renal and glomerular tissue by scar tissue might “dilute out” AT$_1$ receptor-expressing cells. Consistently low AT$_1$ receptor mRNA expression even in patients with incipient renal failure and little or no scarring detected by renal biopsy (Waldherr, personal communication) argues against this possibility.

Another confounding variable is infiltration of the kidney and the glomeruli by nonresident cells, e.g., monocytes and macrophages. The study in diabetic nephropathy was performed primarily because infiltration of glomeruli by nonresident cells is known to be very low in this disease (27). Expression of AT$_1$ receptor mRNA was low in both more and less inflammatory glomerular diseases, i.e., various types of GN, and in noninflammatory glomerular disease, i.e., diabetic nephropathy. This finding argues against a major confounding effect of passenger cells when AT$_1$ mRNA is expressed per unit weight of RNA. Low AT$_1$ receptor mRNA levels in diabetic patients is in agreement with experimental studies in streptozotocin-diabetic rats, where a decrease in AT$_1$ receptor gene expression and angiotensin binding was noted (28).

Our study was limited to assessment of AT$_1$ receptor mRNA. We acknowledge that the overall level of AT$_1$ receptor protein expression and of AngII-mediated events may not be accurately reflected by AT$_1$ receptor mRNA levels alone. RNA stability, posttranslational processing, changes in receptor cycling, and second-messenger uncoupling of AT$_1$ receptors may modulate signal transduction. A reasonably close relationship between AT$_1$ receptor mRNA on the one hand and AngII binding on the other is suggested, however, by parallel changes in levels of AT$_1$ receptor mRNA and AngII binding (29,30).
We are aware that regulation of AT₁ receptors in tissues is complex. AngII has been shown to lower glomerular AT₁ receptor levels in kidney preparations. AngII infusion significantly reduced AngII binding site levels in the rat glomerulus. Mesangial cells showed decreased AT₁ receptor mRNA levels when exposed to AngII (29,30), although different findings were reported for tubular epithelial cells (31). It is therefore tempting to speculate that a decrease in AT₁ receptor mRNA expression to high intrarenal AngII concentrations, provoking a negative feedback response, i.e., AT₁ receptor downregulation.

In summary, low AT₁ receptor mRNA expression was found in glomeruli of patients with inflammatory or noninflammatory glomerular disease. This finding is consistent with an adaptive response of AT₁ receptor expression to high intrarenal AngII levels. We admit, however, that alternative explanations are not excluded.

References
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glomerular mesangial cells. *Biochem Biophys Res Commun* 185: 142–146, 1992


