ETA Receptor Blockade Induces Tubular Cell Proliferation and Cyst Growth in Rats with Polycystic Kidney Disease

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Abstract. Tissue concentrations of ET-1 are markedly elevated in the kidneys of Han:Sprague-Dawley (Han:SPRD) rats, a model of human autosomal dominant polycystic kidney disease (ADPKD). This study analyzed whether disease progression might be attenuated by endothelin receptor antagonists. Heterozygous Han:SPRD rats received an ETA receptor antagonist (LU 135252), a combined ETA/ETB receptor antagonist (LU 224332), or placebo for 4 mo. Glomerulosclerosis, protein excretion, and GFR remained unchanged, whereas interstitial fibrosis was enhanced by both compounds. BP was not reduced by both compounds in Han:SPRD rats. Renal blood flow (RBF) decreased in ADPKD rats treated with the ETA receptor antagonist. Long-term ETA receptor blockade furthermore increased markedly the number of renal cysts (ADPKD rats, 390 ± 119 [cysts/kidney section ± SD]; LU 135252-treated ADPKD rats, 1084 ± 314; P < 0.001), cyst surface area (ADPKD rats, 7.97 ± 2.04 [% of total section surface ± SD]; LU 135252-treated ADPKD rats, 33.83 ± 10.03; P < 0.001), and cell proliferation of tubular cells (ADPKD rats, 42.2 ± 17.3 [BrdU-positive cells/1000 cells]; LU 135252-treated ADPKD rats, 339.4 ± 286.9; P < 0.001). The additional blockade of the ETB receptor attenuated these effects in Han:SPRD rats. Both endothelin receptor antagonists had no effect on BP, protein excretion, GFR, and kidney morphology in Sprague-Dawley rats without renal cysts. It is concluded that ETA receptor blockade enhances tubular cell proliferation, cyst number, and size and reduces RBF in Han:SPRD rats. This is of major clinical impact because endothelin receptor antagonists are upcoming clinically used drugs.

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic hereditary kidney disease in humans, which is seen in about 1 in 1000 live births. This disease accounts for up to 10% of all patients requiring renal replacement therapy. Cysts arise from renal tubular segments as focal areas of dilatation. They progressively enlarge with age and might separate from the nephron of origin. Mutations in two genes, PKD1 and PKD2, are associated with this disease in humans (1,2). Progression of ADPKD, resulting in renal failure, varies between affected members of different families, as well as between different members of the same family. In some individuals, kidney cysts are present in early childhood and progress to end-stage renal failure before age of 40 yr, whereas renal function remains unimpaired throughout lifespan in others. Several general factors, such as gender, germ-line mutations, modifier genes, and epigenetic factors, affect disease progression with respect to cyst growth, interstitial inflammation, and progressive fibrosis (3–5). The renal endothelin system seems to be one of these disease-modifying factors. ET-1 transgenic mice develop small cortical kidney cysts (10,31). It was furthermore shown that the renal ET system is markedly activated in patients with autosomal dominant polycystic disease (ADPKD) (6–8), in polycystic kidneys of cpk mice (9) as well as in Han:SPRD rats (7) (a rat model of ADPKD). The Han:SPRD rat strain develops a form of progressive gender-dependent disease that appears similar in many respects to that seen in the ADPKD in humans (11), even through different genetic defects. The gene responsible for this disease in rats is located on rat chromosome 5 and is not related to the human PKD1 or PKD2 gene (12). ADPKD in humans as well as in Han:SPRD rats is characterized by structural alterations of the kidneys, such as thickening of the tubular basement membrane, interstitial fibrosis, and formation of cysts leading to end-stage kidney disease (11). Tubular cell proliferation seems to be an important step during pathogenesis of kidney cysts in ADPKD, because the main feature of the disease is the development of renal cysts, first occurring in the proximal tubules, and with time dominating all segments of the nephron. It is known that a primarily activated renal endothelin sys-
system causes renal scarring/fibrosis (10); we therefore hypothesized that the activated endothelin system in ADPKD contributes to the progression of kidney fibrosis seen in these rats. Thus, a long-term pharmacologic blockade of the endothelin system might be a new approach to reduce disease progression in ADPKD. We tested this hypothesis in Han:SPRD rats using endothelin receptor antagonists that are currently clinically tested in large clinical trials with regard to heart failure and pulmonary hypertension.

### Materials and Methods

#### Animals and Study Design
Male heterozygous (cy/+) Han:SPRD rats (5) were analyzed. The animals were maintained as an inbred colony in the Department of

### Table 1. Body weight, BP, kidney weight, GFR, renal morphometric data, and clinical chemistry of Sprague-Dawley rats after 4 mo of treatment with ETA or combined ETA/ETB receptor antagonists

<table>
<thead>
<tr>
<th></th>
<th>Sprague-Dawley Rats</th>
<th>Sprague-Dawley Rats + LU 135252</th>
<th>Sprague-Dawley Rats + LU 224332</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>509.9 ± 43.0</td>
<td>518.0 ± 29.8</td>
<td>516.9 ± 50.7</td>
</tr>
<tr>
<td>Kidney weight (%) of body weight</td>
<td>0.63 ± 0.09</td>
<td>0.64 ± 0.11</td>
<td>0.62 ± 0.09</td>
</tr>
<tr>
<td>Mean arterial BP</td>
<td>93.4 ± 9.1</td>
<td>92.3 ± 9.5</td>
<td>94.2 ± 10.9</td>
</tr>
<tr>
<td>GFR (ml/24 h per 100 g BW)</td>
<td>2198 ± 348</td>
<td>2221 ± 432</td>
<td>2087 ± 378</td>
</tr>
<tr>
<td>Glomerulosclerosis score</td>
<td>1.70 ± 0.31</td>
<td>1.67 ± 0.23</td>
<td>1.60 ± 0.32</td>
</tr>
<tr>
<td>Interstitial fibrosis (% fibrotic area)</td>
<td>2.84 ± 1.94</td>
<td>2.65 ± 1.01</td>
<td>2.97 ± 0.87</td>
</tr>
<tr>
<td>Plasma protein (g/dl)</td>
<td>5.7 ± 0.5</td>
<td>5.5 ± 0.4</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>Plasma albumin (g/dl)</td>
<td>2.5 ± 0.2</td>
<td>2.4 ± 0.3</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>1.3 ± 1.0</td>
<td>1.4 ± 0.9</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>Serum urea (mg/dl)</td>
<td>28.4 ± 9.8</td>
<td>29.1 ± 10.2</td>
<td>31.2 ± 12.0</td>
</tr>
<tr>
<td>Serum alanine aminotransferase (U/L)</td>
<td>14.6 ± 4.0</td>
<td>14.2 ± 4.1</td>
<td>14.5 ± 3.9</td>
</tr>
<tr>
<td>Urinary protein excretion (g/24 h)</td>
<td>0.03 ± 0.03</td>
<td>0.03 ± 0.05</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>Urinary albumin excretion (g/24 h)</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.02</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

* Sprague-Dawley rats (8-wk-old) were treated for 4 months with placebo, LU 135252 (ETA receptor antagonist), or LU 224332 (combined ETA/ETB receptor antagonist), respectively. Urine (24-h urine in a metabolic cage) and blood were taken in the last treatment week. Data are given as means ± SD. None of the parameters tested were significantly influenced by LU 135252 or LU 224332 treatment.

### Table 2. Body weight clinical chemistry of HanSPRD rats after 4 mo of treatment with ETA or combined ETA/ETB receptor antagonists

<table>
<thead>
<tr>
<th></th>
<th>HanSPRD Rats</th>
<th>HanSPRD Rats + LU 135252</th>
<th>HanSPRD Rats + LU 224332</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>402.9 ± 24.0</td>
<td>350.7 ± 37.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>433.2 ± 33.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney weight (%) of body weight</td>
<td>1.22 ± 0.04</td>
<td>3.10 ± 0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>Mean arterial BP (mmHg)</td>
<td>92.9 ± 8.5</td>
<td>101.9 ± 15.4</td>
<td>106.5 ± 12.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GFR (ml/24 h per 100 g BW)</td>
<td>475 ± 143</td>
<td>429 ± 132</td>
<td>356 ± 82</td>
</tr>
<tr>
<td>Glomerulosclerosis score</td>
<td>2.22 ± 0.30</td>
<td>2.49 ± 0.22</td>
<td>2.09 ± 0.20</td>
</tr>
<tr>
<td>Interstitial fibrosis (% fibrotic area)</td>
<td>15.6 ± 3.8</td>
<td>21.1 ± 2.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.3 ± 3.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma protein (g/dl)</td>
<td>5.0 ± 0.5</td>
<td>5.5 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.7 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma albumin (g/dl)</td>
<td>2.4 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>2.1 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>2.7 ± 1.1</td>
<td>1.8 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma urea (mg/dl)</td>
<td>78.4 ± 17.4</td>
<td>105.0 ± 43.2</td>
<td>101.2 ± 13.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma alanine aminotransferase (U/L)</td>
<td>12.6 ± 3.0</td>
<td>15.0 ± 3.4</td>
<td>14.8 ± 3.7</td>
</tr>
<tr>
<td>Urinary protein excretion (g/24 h)</td>
<td>0.05 ± 0.03</td>
<td>0.08 ± 0.04</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Urinary albumin excretion (g/24 h)</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> HanSPRD rats were treated for 4 mo with placebo, LU 135252 (ETA receptor antagonist), or LU 224332 (combined ETA/ETB receptor antagonist), respectively. Urine (24-h urine in a metabolic cage) and blood were taken in the last treatment week. Data are given as means ± SD.

<sup>b</sup> P < 0.05 versus HanSPRD rats.

<sup>c</sup> P < 0.01 versus HanSPRD rats.

<sup>d</sup> P < 0.05 versus LU 135252-treated HanSPRD rats.
Breeding of Han:SPRD rats was performed by mating heterozygous (cy/H11001) Han:SPRD rats. Discrimination between heterozygous (cy/H11001) Han:SPRD rats and healthy (H11001/H11001) Sprague Dawley rats was done by analyzing serum urea and creatinine concentrations. These tests allowed a reliable discrimination between heterozygous (cy+/+) Han:SPRD rats and healthy (+/+) Sprague Dawley rats was done by analyzing serum urea and creatinine concentrations. These tests allowed a reliable discrimination between heterozygous (cy+/+) Han:SPRD rats and healthy (+/+) Sprague Dawley rats.

Figure 1. Typical kidney sections from Han:Sprague-Dawley (Han:SPRD) rats after 4 mo of treatment with the ETA receptor antagonist or the combined ETA/ETB receptor antagonist: (A) nontreated Han:SPRD rat; (B) LU 135252 (ETA receptor antagonist)–treated Han:SPRD rat; (C) LU 224332 (combined ETA/ETB receptor antagonist). Magnification is the same in all panels; the bar in panel marks 1 mm. Bar graphs show number of renal cysts (D) and cyst surface area (E) in Han:SPRD rats after 4 mo of treatment with the ETA receptor antagonist LU 135252 or the combined ETA/ETB receptor antagonist LU 224332. Data are given as mean ± SD. **P < 0.01 compared with nontreated Han:SPRD rats; ***P < 0.001 compared with nontreated Han:SPRD rats.
rats and healthy (+/+) Sprague Dawley rats if the rats were older than 7 wk. The Han:SPRD rats were fed a commercial diet (Altromin®, Altromin GmbH, Germany) and given water ad libitum. We established three groups:

- A placebo-treated group with heterozygous (cy/) Han:SPRD rats, \( n = 12 \)
- A group of heterozygous (cy/) Han:SPRD rats treated with the ETA receptor antagonist LU 135252 (50 mg/kg per d), \( n = 10 \)
- A group of heterozygous (cy/) Han:SPRD rats treated with the combined ETA/ETB receptor antagonist LU 224332 (B) and also the combined ETA/ETB receptor antagonist LU 224332 (C). Sections were stained with Sirius red. Connective tissue appears red after Sirius red staining.

**Figure 2.** Renal blood flow (A) and renal vascular resistance (B) in Han:SPRD rats after 4 mo of treatment with the ETA receptor antagonist or the combined ETA/ETB receptor antagonist. Data are given as mean ± SD. **# #** \( P < 0.01 \) compared with nontreated Han:SPRD rats; **##** \( P < 0.01 \) compared with LU 224332–treated Han:SPRD rats.

**Figure 3.** Typical kidney section of a nontreated 6-mo-old Han:SPRD rat (A). Interstitial fibrosis was enhanced after 4 mo of treatment with the ETA receptor antagonist LU 135252 (B) and also the combined ETA/ETB receptor antagonist LU 224332 (C). Sections were stained with Sirius red. Connective tissue appears red after Sirius red staining.
Table 3. Cell proliferation in kidneys of HanSPRD rats after 4 mo of treatment with ETA or combined ETA/ETB receptor antagonists

<table>
<thead>
<tr>
<th></th>
<th>HanSPRD Rats</th>
<th>HanSPRD Rats + LU 135252</th>
<th>HanSPRD Rats + LU 224332</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular cell proliferation (BrdU-positive cells/1000 cells)</td>
<td>5.5 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.4 ± 10.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.9 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interstitial cell proliferation (BrdU-positive cells/1000 cells)</td>
<td>16.9 ± 8.0</td>
<td>26.4 ± 13.9</td>
<td>16.5 ± 3.5</td>
</tr>
<tr>
<td>Tubular cell proliferation (BrdU-positive cells/1000 cells)</td>
<td>42.2 ± 17.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>339.4 ± 286.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>123.9 ± 19.5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epithelial cyst wall cell proliferation (BrdU-positive cells/1000 cells)</td>
<td>9.1 ± 6.4</td>
<td>7.2 ± 4.0</td>
<td>8.2 ± 1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cell proliferation was analyzed by in vivo BrdU incorporation. Data are given as means ± SD.

<sup>b</sup> P < 0.05 compared with glomerular, interstitial, and cyst wall epithelial cells of nontreated Han:SPRD rats.

<sup>c</sup> P < 0.05 compared with the same cell type of nontreated heterozygous (cy/) Han:SPRD rats.

<sup>d</sup> P < 0.01 compared with the same cell type of nontreated heterozygous (cy/) Han:SPRD rats.

<sup>e</sup> P < 0.001 compared with the same cell type of nontreated heterozygous (cy/) Han:SPRD rats.

- A group of heterozygous (cy/) Han:SPRD rats treated with the combined ETA/ETB receptor antagonist LU 224332 (50 mg/kg per d), n = 5

To control for potential toxic effects of the endothelin receptor antagonists, we furthermore established the following three groups:

- A placebo-treated group with Sprague-Dawley rats, n = 10
- A group of Sprague-Dawley rats treated with the ETA receptor antagonist LU 135252 (50 mg/kg per d), n = 10
- A group of Sprague-Dawley rats treated with the combined ETA/ETB receptor antagonist LU 224332 (50 mg/kg per d), n = 10

We used Sprague-Dawley rats as controls, because the genetic background of the Han:SPRD rats are Sprague-Dawley rats.

Treatment with the endothelin receptor antagonists (13) started in 8-wk-old male heterozygous (cy/) Han:SPRD rats or male Sprague-Dawley rats of the same age. The animals were treated for 4 mo. Drugs were given orally within the food. At the end of the drug treatment period, all rats were placed into a metabolic cage for 24 h. Urine was collected, and blood was taken at the end of the urine-collection period. All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

**Surgery**

Anesthesia was introduced and maintained by 1 g/kg body wt urethane intraperitoneally (Sigma). The animals were then placed on a thermostated table to maintain normal body temperature. After an incision in the left groin, a polyethylene catheter (ID, 0.54 mm; OD, 0.96 mm with narrowed tip) was implanted into the left femoral artery in such a way that the tip was placed distally to both renal arteries. Another catheter of the same dimensions was inserted into the femoral vein. The latter line was used to continuously infuse a warmed (37°C) solution of 0.9% NaCl (10 ml/h per kg) throughout the surgery and the following experiment. The abdominal cavity was then opened, and the left renal artery and vein were sealed. An ultrasound transit time flow probe placed around the renal artery (ID, 1 mm). For further details, see reference 14. Renal vascular resistance (RVR) was calculated using the following formula: $RVR = (BP-20 mmHg)/RBF$. The offset of 20 mmHg is an adjustment for the so-called waterfall phenomenon of the kidney.

**GFR and Clinical Chemistry**

Serum concentrations of alanine aminotransferase, creatinine, protein, and albumin and urine concentrations of creatinine, protein, and albumin were determined by using the appropriate kits in an automatic analyzer in the department of clinical biochemistry and laboratory medicine of the Charité University hospital. The absolute excretion of proteins was calculated by the equation: $Ux × V24$, where $Ux$ is the concentration of protein in urine, and $V24$ is the amount of urine excreted in 24 h. The endogenous creatinine clearance was calculated using the formula $C = Uc × Uvol/Sc$, where $C$ is creatinine clearance, $Uc$ is urinary creatinine concentration, $Uvol$ is urine volume, and $Sc$ is serum creatinine concentration.

**Histologic Evaluation**

For pathohistologic evaluation, all samples were embedded in paraffin, cut in 3-μm sections, and submitted to hematoxylin-eosin, Sirius red, and periodic acid-Schiff (PAS) staining and analyzed as recently described (15). The severity of interstitial matrix deposition was evaluated after Sirius red staining using a computer-aided image-analyzing system. We measured the relationship of red stained interstitial area (connective tissue) to total interstitial area of the whole kidney section. Interstitial area was defined as total section area minus cyst area minus glomerular area minus area of blood vessels. Glomerulosclerosis was defined by the presence of PAS-positive material within the glomeruli. To consider differences in the degree of glomerulosclerosis, a semiquantitative scoring system was used as recently described (15). All tissue samples for scoring were evaluated independently by two investigators without prior knowledge of the group to which the rats belonged.

**In Vivo BrdU Incorporation**

*In vivo* BrdU incorporation was performed exactly as recently described (16). Briefly, we injected BrdU intraperitoneally to label the DNA in vivo 24 h before sacrificing the rats. Paraffin sections were dewaxed and enzymatically digested with trypsin. After washing, the sections were treated with 4 M HCl followed by a neutralizing step. The probes were subsequently incubated with anti-BrdU-AP antibody solution. Substrate reaction was then started with a sufficient amount of the freshly prepared substrate solution and incubated at room...
temperature for 20 min until a clearly visible color developed. The slides were washed and embedded with glycerine/PBS. We counted BrdU-positive cells and the total cell number to calculate a proliferation index (BrdU-positive cells/total cell number). Cell proliferation was analyzed separately in the glomeruli, tubular cells, blood vessels, interstitial cells, and epithelial cells of the cystic wall.

Detection of Apoptotic Cells

To evaluate apoptotic cells, we used a combined TUNEL double-staining method (Roche, Mannheim, Germany) as recently described (17). Briefly, paraffin kidney sections were incubated with digoxigenin-dUTP in the presence of TdT. TUNEL-positive cells were visualized by anti-digoxigenin FITC-conjugated F(ab’prime)2 fragments. Counterstaining was performed using DAPI dye (1 μg/ml methanol) in a subsequent incubating step. Finally, sections were mounted using VectaShield (Vector Laboratories, Burlingame, VT). Spleen served as positive control. Negative controls for TUNEL staining were made by omitting TdT, according to the manufacturer’s protocol.

Statistical Analysis

The Mann-Whitney U test was used to compare groups. The level of significance was set at P < 0.05. Statistical analysis was performed using SPSS for Windows, Version 11.0.

Results

Long-term treatment with LU 135252 and LU 224332 had no nonspecific or toxic side effects. All Sprague-Dawley rats (the genetic background of the Han:SPRD rats are Sprague-Dawley rats) survived. They grew well and were healthy (Table 1). Light microscopy of the kidneys revealed completely normal kidney morphology with no abnormalities within the tubules. Cysts were not detectable in LU 135252-treated and LU 224332-treated Sprague-Dawley rats.

The heterozygous (cy/+) Han:SPRD rats also tolerated treatment with both endothelin receptor antagonists (LU 135252 or LU 224332) without adverse events. No toxic side effects (elevated liver enzymes, infectious diseases, clinical signs of diseases, or increased mortality) were seen in the LU 135252-treated or LU 224332-treated ADPKD rats. We observed differences with respect to serum protein and C-reactive protein between nontreated and treated Han:SPRD rats; however, all the values were within the normal range for these parameters for rats in our laboratory. We thus suggest that these differences, although statistically significant, are of minor pathophysiologic impact. The body weight of rats treated with the ETA receptor antagonist, on the other hand, was significantly lower as compared with nontreated ADPKD rats (Table 2). Treatment with the endothelin receptor antagonists affected neither urinary total protein excretion nor urinary albumin excretion (Table 2).

ETA receptor antagonist–treated ADPKD rats had a markedly increased kidney weight, increased number of renal cysts, and increased cyst surface area (Table 2 and Figure 1). The additional blockade of the ETB receptor using the combined ETA/ETB receptor antagonist LU 224332 blunted the effects on kidney weight, number of renal cysts, and cyst surface area as compared with a sole ETA blockade (Table 2 and Figure 1).

GFR determined by the endogenous creatinine clearance was slightly lower in heterozygous (cy/+) Han:SPRD rats after 4 mo of treatment with the ETA receptor antagonist and the combined ETA/ETB receptor antagonist (Table 2). However, these differences were statistically NS. Mean arterial BP was significantly elevated in heterozygous (cy/+) Han:SPRD rats treated for 4 mo with the combined ETA/ETB receptor antagonist LU 224332 (Table 2).

Total renal blood flow (RBF), measured at the arteria renalis, decreased markedly in LU 135252–treated heterozygous (cy/+) Han:SPRD rats as compared with nontreated and LU 224332–treated ADPKD rats (Figure 2A). The ETA antagonist LU 135252 caused a sustained increase of renal vascular resistance (RVR) (Figure 2B), because BP did not fall in this
Apoptotic cells in kidneys of Han:SPRD rats after 4 mo of treatment with ETA or combined ETA/ETB receptor antagonists

<table>
<thead>
<tr>
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<th>HanSPRD Rats + LU 135252</th>
<th>HanSPRD Rats + LU 224332</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic glomerular cells (apoptotic cells/10000 cells)</td>
<td>0.61 ± 1.29</td>
<td>0.85 ± 1.56</td>
<td>0.61 ± 0.84</td>
</tr>
<tr>
<td>Apoptotic interstitial cells (apoptotic cells/10000 cells)</td>
<td>0.21 ± 0.18</td>
<td>0.32 ± 0.33</td>
<td>0.21 ± 0.13</td>
</tr>
<tr>
<td>Apoptotic tubular cells (apoptotic cells/10000 cells)</td>
<td>0.06 ± 0.08</td>
<td>2.23 ± 1.87</td>
<td>0.06 ± 0.13</td>
</tr>
<tr>
<td>Apoptotic epithelial cyst wall cells (apoptotic cells/10000 cells)</td>
<td>1.05 ± 1.81</td>
<td>0.23 ± 0.41</td>
<td>0.92 ± 0.58</td>
</tr>
</tbody>
</table>

* Apoptosis was analyzed by a combined TUNEL double-staining method. Data are given as means ± SD.

b $P < 0.001$ compared with the same cell type of nontreated heterozygous (cy/+ ) Han:SPRD rats.

Discussion

The endothelin system is activated in Han:SPRD rats as well as in humans with ADPKD (7). The reasons for this activation are presently unknown. The known genetic defects in patients with ADPKD as well as in the rat model of ADPKD analyzed in this study are not related to genes of the endothelin system (1,2,12). However, it was suggested that cyst growth causes focal ischemia (18); ischemia on its own, via an induction of hypoxia-inducible factors (19,20), is one of the most potent stimuli of the endothelin system in vivo (21). Given this potential pathway of activating the endothelin system in ADPKD, long-term treatment studies with endothelin receptor antagonists using Han:SPRD rats are most probably of major impact also for the human disease.

A blockade of especially the ETA receptor induces a marked increase of kidney weight, increased number of renal cysts, and increased cyst surface area by comparison of absolute numbers) negligible increase in tubular cell apoptosis has only a minor impact on the ETA receptor–induced alterations in tubular cell turnover in Han:SPRD rats. To our knowledge, this is the first study showing that long-term blockade of the ETA receptor increases cell proliferation in vivo. The additional blockade of the ETB receptor seems to attenuate the effects of a sole ETA receptor blockade. Induction of growth/cell proliferation by blocking the ETA receptor is only seen in the polycystic kidney; other organs of these ADPKD rats are not affected (data not shown). Neither kidney function nor kidney morphology was altered by both endothelin receptor antagonists in healthy Sprague-Dawley rats (the genetic background of the Han:SPRD rats).

Blocking the ETA receptor usually decreases cell proliferation. The ETA receptor antagonist used in our study (LU 135252) decreases cell proliferation and kidney fibrosis in various models of progressive kidney failure/fibrosis like diabetic nephropathy (13), chronic renal allograft rejection (22,23), and rats with surgical renal mass ablation (24). ETA receptor antagonism was furthermore shown to inhibit prostate cancer cell proliferation (25). Given the strong evidence that ETA receptor antagonists usually inhibit cell prolifer-
Our finding of a marked increase of kidney weight and especially tubular cell proliferation, measured by the in vivo BrdU incorporation method, is striking. Three points should be considered regarding this unexpected finding.

1. Blocking the ETA receptors might direct ET-1 toward the ETB receptor. The ETB receptor is especially localized on renal tubular cells (26,27). Tubular cell proliferation is an important step during pathogenesis of kidney cysts in ADPKD. The main feature of the disease is the development of renal cysts. They develop in young ADPKD rats in the proximal tubules and, with time, dominate all segments of the nephron (28,29). We suggest that ET-1 may further enhance the already augmented tubular cell proliferation via the tubular ETB receptor. This hypothesis is supported by the finding that the additional blockade of the ETB receptor ameliorated the effects of a sole ETA blockade. The fact that LU 224332 could not completely abolish the effects of a sole ETA blockade by LU 135252 is most probably due to the pharmacologic profile of LU 224332. LU 224332 has a $K_i$ for the ETA receptor of 3.5 nmol/L and a $K_i$ for the ETB receptor of 7.2 nmol/L (30), meaning that LU 224332 blocks the ETA receptor somewhat better than the ETB receptor. In agreement with the above-described concept (ET-1 promotes cyst growth via the tubular ETB receptor) is the finding that ET-1 transgenic mice develop renal cysts (10,31).

2. It is also possible that the ETA blockade induced reduction of renal blood flow in ADPKD rats might cause an enhanced expression of hypoxia inducible factors (HIF) in tubular cells (20,21). HIF might exert further proliferation-stimulating effects on the already proliferating tubular cells in ADPKD rats.

3. The endothelin receptors are G-protein–coupled receptors. G-protein–coupled receptors are thought to contribute to the progression of PKD through the generation of cAMP (32). It is interesting that inhibition of this receptor pathway in ADPKD rats does not inhibit the disease process. This may suggest that only specific G-protein receptors contribute to the progression of PKD and that a generalized inhibition of G protein receptor pathways could augment the disease process.

Treatment with both endothelin receptor antagonists (LU 135252 and LU 224332) causes a moderate increase of interstitial fibrosis in heterozygous (cy+/+) Han:SPRD rats. Again, this was not expected given that blocking the renal endothelin system is usually a powerful antifibrotic strategy in experimental models of chronic progressive kidney fibrosis (13,22–24). Thus, increased fibrosis after blocking the endothelin system in polycystic kidneys is more likely related to intrarenal endothelin-dependent hemodynamic factors leading to an impaired renal microcirculation. Total RBF was reduced in LU 135252–treated ADPKD rats and not altered in LU 224332–treated ADPKD rats. This implies that blood flow per gram kidney weight is reduced after blocking the endothelin system in both treatment groups, because total kidney weight of the LU 135252–treated and LU 224332–treated ADPKD rats was markedly elevated. The reduced relative blood flow, considering kidney weight (see above), in heterozygous (cy+/+) Han:SPRD rats after blocking the ET system would lead to a further increase of

![Figure 5. Typical kidney section of nontreated ADPKD rats showing epithelial cyst wall cells apoptosis (A), tubular cell apoptosis (B), and glomerular cell apoptosis (C). Detection of apoptotic cells was done by a combined TUNEL double-staining method (see Materials and Methods). Cy, kidney cyst; Tu, renal tubule; Gl, glomerulus; * apoptotic cells. The ETA receptor antagonist LU 135252 increased tubular cell apoptosis, but it did not influence apoptosis in glomerular cells and epithelial cyst wall cells (see also Table 4).](image)
the already existing cyst growth–related focal ischemia in ADPKD rats (18). Ischemia on its own is a well-known pro-fibrotic stimulus (for review, see reference 33). A very recent study also demonstrates that long-term treatment with an ETA receptor antagonist in rats with two kidney-one clip (2K-1C) renovascular hypertension increases fibrosis in the clipped (ischemic) kidney (15).

The combined ETA/ETB receptor antagonist led to a mild increase in mean arterial BP as compared with nontreated ADPKD rats. Beside the effects on kidney fibrosis and cyst growth, the combined ETA/ETB receptor antagonist might have additional effects, via the ETB receptor, on renal water and salt excretion in ADPKD rats. It was recently shown that the complete absence of a functional ETB receptor in rats may cause salt-sensitive elevation of BP due to an enhanced tubular salt reuptake (16,34).

In conclusion, our study indicates that the activated endothelin system in heterozygous (Cy+/+) Han:SPRD rats is substantially involved in the regulation of tubular cell proliferation and cyst growth (cyst size and number). It also plays an important role in the development of interstitial fibrosis. Thus, the endothelin system is a major disease-modifying system in ADPKD. This is of clinical impact, because endothelin receptor antagonists will become new clinically used drugs in near future. On the basis of our data, especially sole ETA receptor antagonists but also combined ETA/ETB receptor antagonists might be harmful for patients with ADPKD.

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