Diabetic Endothelin B Receptor–Deficient Rats Develop Severe Hypertension and Progressive Renal Failure

Thiemo Pfab,*† Christa Thöne-Reinke,* Franziska Theilig,‡ Ines Lange,* Henning Witt,*§ Christiane Maser-Gruth,‖ Michael Bader,‖ Johannes-Peter Stasch,** Patricia Ruiz,**§ Sebastian Bachmann,‖ Masashi Yanagisawa,†† and Berthold Hocher*

*Center for Cardiovascular Research/Institute of Pharmacology and †Institute of Anatomy, Charité Mitte, ‡Department of Nephrology, Charité Campus Benjamin Franklin, §Max Planck Institute for Molecular Genetics, and ‖Max-Delbrück Center for Molecular Medicine, Berlin, Germany; ††Institute of Pharmacology, University of Heidelberg, Heidelberg, Germany; **Bayer AG, Wuppertal, Germany; and ‡‡Howard Hughes Medical Institute, University of Texas, Dallas, Texas

The endothelin (ET) system has been implicated in the pathogenesis of diabetic nephropathy. The role of the ET-B receptor (ETBR) is still unclear. The effect of ETBR deficiency on the progression of diabetic nephropathy in a streptozotocin model was analyzed in four groups: (1) Homozygous ETBR-deficient (ETBRd) diabetic rats, (2) ETBRd rats, (3) diabetic controls, and (4) wild-type controls. BP and kidney function were measured for 10 wk, followed by biochemical and histologic analysis of the kidneys. The study demonstrates that ETBRd diabetic rats on a normal-sodium diet develop severe hypertension, albuminuria, and a mild reduction of creatinine clearance. The strong BP rise seems not to be caused by activation of the renin-angiotensin-aldosterone system or by suppression of the nitric oxide system. Elevated plasma ET-1, possibly reflecting reduced ETBR activity, is suggested as the cause of ESRD and dialysis treatment in industrialized countries (1,2). Strategies to stop its progression are still not effective enough. The endothelin (ET) system seems to be particularly important for the pathogenesis of diabetic nephropathy: (1) The renal ET system is activated in patients with diabetic nephropathy as well as in animal models of diabetes-induced kidney damage (8–10), (2) a primary activated renal ET system causes kidney fibrosis in ET-1 transgenic mice (7,11) as well as in ET-2 transgenic rats (12), and (3) blocking the ET system using ET-A receptor (ETAR) or combined ETAR/ETBR antagonists improves protein and albumin excretion and reduces pathologic matrix protein synthesis in diabetic animals in a BP-independent manner (3–6).

There is evidence that the ETBR might play a role in remodeling processes of the kidney (13). This led us to the hypothesis that reduction of ETBR activity might be protective against diabetic nephropathy. A widely used ETBR-deficient (ETBRd) rat model, developed by the group of Yanagisawa (14–16), served as a tool to test this hypothesis. In this study, we analyzed the effect of ETBR deficiency on the progression of diabetic nephropathy in rats with streptozotocin (STZ)-induced diabetes.

Materials and Methods

Animals and Study Protocol

ETBRd rats (ETBRd/sl) and wild-type (WT) controls were provided by Yanagisawa (16). All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals. Genotyping was accomplished by standard PCR of genomic DNA as described previously (15,16). Animals were kept on a standard diet (0.2% sodium) and water ad libitum. ETBRd rats are known to be normotensive on a sodium-deficient diet and mildly hypertensive on a standard diet and exhibit severe hypertension on a high-sodium diet (15). We analyzed four groups of 6-mo-old male rats: Homozygous ETBRd rats with STZ-induced diabetes (ETBRd-STZ; n = 6), homozygous ETBRd nondiabetic rats (ETBRd; n = 9), diabetic controls (STZ; n = 8), and WT controls (n = 6). The animals received a single tail-vein injection of STZ (65 mg/kg) or vehicle as described previously (9). Only STZ-treated animals with plasma glucose concentrations >15 mmol/L after 48 h were included in the study. Systolic BP and heart rate were measured in weeks 0, 5, and 10 by tail plethysmography as described previously (17). All animals were weighed and placed in metabolic cages in weeks 0, 5, and 10. Blood was taken on...
EDTA from the retro-orbital vein plexus. Plasma was obtained after centrifugation (4000 × g, 3 min) and stored at −20°C. The animals were killed after week 10, and heart and kidneys were excised, washed in ice-cold saline, blotted dry, and weighed. The left kidney was frozen immediately in liquid nitrogen. The right kidney and the heart were formalin fixed (4%, 24 h).

**Plasma and Urine Measurements**

Glucose, osmolality, sodium, potassium, creatinine, urea, and albumin were measured using an automated analyzer (Hitachi 717, Boehringer Mannheim, Germany). The endogenous creatinine clearance was used as a surrogate for the GFR. Plasma renin concentration (PRC) and activity (PRA) were determined by an indirect enzyme-kinetic assay based on the generation of angiotensin I according to published methods (18). Measurement of urinary aldosterone was performed by RIA after organic solvent extraction and chromatographic purification on celite columns (19). Urinary excretion of cGMP was determined in triplicate using a commercial RIA kit (IBL, Hamburg, Germany) as described previously (20). Urinary excretion of nitric oxide (NO) metabolites (nitrite and nitrate) was measured using a commercial test kit (Boehringer Ingelheim, Mannheim, Germany). Briefly, nitrate is reduced to nitrite, which then reacts with sulfanilamide and Griess chromophore. The resulting diazo dye is spectrophotometrically quantifiable at 540 nm (21). Measurement of ET-1 concentration in plasma and urine was performed as described previously (22) using a commercially available ELISA-Kit (Immundiagnostik, Bensheim, Germany).

**Histology**

All samples were embedded in paraffin; cut in 3-μm sections; and submitted to periodic acid-Schiff (PAS), elastica, and Sirius red staining. The analysis was performed on the basis of a previous report (7). Glomerulosclerosis was defined by the presence of PAS-positive material within the glomeruli. A semiquantitative score was used to grade a minimal of 80 glomeruli per specimen. A score from 1 to 4 was assigned according to the percentage of PAS-positive material within each glomerulus (<25%, >25%, >50%, and >75%). All samples were evaluated blinded and independently by two investigators (23). A glomerulosclerosis index was calculated by averaging the grades assigned to all glomeruli. The media/lumen ratio of all cross-cut intraglomerular arterioles that were found in one histologic section per kidney was assigned to all glomeruli. The media/lumen ratio of all cross-cut intraglomerular arterioles that were found in one histologic section per kidney was assigned to all glomeruli. The media/lumen ratio of all cross-cut intraglomerular arterioles that were found in one histologic section per kidney was assigned to all glomeruli. The media/lumen ratio of all cross-cut intraglomerular arterioles that were found in one histologic section per kidney was assigned to all glomeruli. The media/lumen ratio of all cross-cut intraglomerular arterioles that were found in one histologic section per kidney was assigned to all glomeruli.

**Immunohistochemistry**

Paraffin sections (3 μm) were dewaxed and rehydrated, and antigen retrieval was carried out by heating in a microwave oven in 2.9 g/L citrate buffer (pH 6.0, 20 min) or by incubating in a pressure cooker (5 min). After blocking in 5% skim milk, sections were incubated with primary rabbit antibodies against renin (gift from Prof. Dr. A. Kurtz, Regensburg, Germany) and neuronal NO synthase (nNOS) (Axxora, Grünberg, Germany) for 2 h at room temperature followed by an overnight incubation. Detection of bound antibodies was performed using biotinylated secondary anti-rabbit antibodies and a catalyzed signal amplification system (DakoCytomation, Hamburg, Germany) based on the streptavidin-biotin-peroxidase reaction, according to the manufacturer’s instructions. A signal was generated by incubation with diaminobenzidine and H2O2. For evaluation of the arteriolar renin status, the number of renin-positive sites at the juxtaglomerular apparatus and at upstream locations in preglomerular vessels was determined within an area of 100 glomeruli. The evaluation was based on the well-established fact that, with varying stimuli, a metaplastic transformation occurs between renin-containing and typical smooth muscle cells of the afferent arteriolar wall, thereby displaying a length shift of the immunoreactive portion of the vessel in an up- or downstream direction of the blood flow (24). The changes correspond to the levels of renal renin synthesis and to plasma renin levels under various conditions (24). The amount of renin mRNA and immunoreactive renin-containing arterioles are known to be largely co-localized and to vary in parallel (25). Quantification of nNOS was performed similarly, as described previously (25).

**Western Blot**

The frozen left kidney was pulverized in liquid nitrogen and then homogenized by ultrasonic homogenizer (20 s) in ice-cold buffer (1 μl/mg) that contained 250 mmol/L sucrose, 10 mmol/L triethanolamine, and 1 tablet of protease inhibitor cocktail per 50 ml (Roche, Grenzach-Wyhlen, Germany). The homogenate was centrifuged (4000 × g, 4°C, 10 min), and the supernatant was ultracentrifuged to obtain a fraction enriched for membranes (200,000 × g, 4°C, 60 min). The pellet was resuspended and stored at −80°C. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL), and the samples were diluted accordingly with modified Laemml buffer to ensure equal loading. Membrane proteins (25 μg/lane) were separated by SDS-PAGE (10%) and wet-blotted onto nitrocellulose membranes. Coomassie staining of gels and Ponceau staining of membranes confirmed equal loading of proteins. Membranes were blocked with 5% skim milk (60 min) and then incubated overnight (4°C) with primary rabbit antibodies directed against ETBR (1:200; gift from Prof. Dr. W. Müller-Esterl, Frankfurt, Germany), the α subunit of the epithelial sodium channel (ENaC, 1:200, Biotrend, Köln, Germany), and primary sheep antibodies against ETAR (1:200; Axxora, Grünberg, Germany). After extensive washing, blots were incubated with a horseradish peroxidase–linked anti-rabbit (anti-sheep for ETAR) IgG (60 min, 1:3000; DakoCytomation). Immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham Pharmacia, Freiburg, Germany) and were subsequently quantified with the Bio-Profile Bio-1D 97.04 software (Froebel, Wasserburg, Germany).

**Statistical Analyses**

Data were analyzed with SPSS 11.5 (SPSS, Inc., Chicago, IL). Results are expressed as mean ± SD. Differences between groups were compared by the nonparametric Kruskal-Wallis and the Mann-Whitney U tests. All tests were two-sided, and P < 0.05 was considered significant.

**Results**

**Statistical Analyses**

Data were analyzed with SPSS 11.5 (SPSS, Inc., Chicago, IL). Results are expressed as mean ± SD. Differences between groups were compared by the nonparametric Kruskal-Wallis and the Mann-Whitney U tests. All tests were two-sided, and P < 0.05 was considered significant.

**Basic Characterization of the Model**

As expected, a strong rise of plasma glucose and osmolality, urinary glucose excretion, fluid intake/excretion, weight loss, and bradycardia is observed after the induction of diabetes, independent of the genotype (Table 1).

**BP**

As described previously (15) ETBRd animals are hypertensive when on a normal-salt diet. Baseline systolic BP levels are 164 ± 12 mmHg in ETBRd versus 131 ± 13 mmHg in WT rats.
Table 1. Plasma/urine glucose, osmolality, fluid balance, body weight, and heart rate (week 10 after induction of diabetes)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ETBRd-STZ</th>
<th>ETBRd</th>
<th>STZ</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose plasma (mmol/L)</td>
<td>39.0 ± 7.0b</td>
<td>10.0 ± 0.5</td>
<td>34.0 ± 2.7b</td>
<td>10.0 ± 1.0</td>
</tr>
<tr>
<td>Glucose urine (mmol/d)</td>
<td>17.0 ± 8.0b</td>
<td>0.005 ± 0.002</td>
<td>16.0 ± 6.0b</td>
<td>0.007 ± 0.002</td>
</tr>
<tr>
<td>Osmolality plasma (mmol/kg)</td>
<td>336.0 ± 9.5b</td>
<td>302.0 ± 2.1</td>
<td>328.0 ± 8.6b</td>
<td>302.0 ± 3.4</td>
</tr>
<tr>
<td>Fluid intake (ml/d)</td>
<td>56.0 ± 28.0c</td>
<td>21.0 ± 10.0</td>
<td>61.0 ± 11.0b</td>
<td>19.0 ± 4.1</td>
</tr>
<tr>
<td>Urine volume (ml/d)</td>
<td>52.0 ± 19.0b</td>
<td>18.0 ± 8.5</td>
<td>46.0 ± 8.9b</td>
<td>16.0 ± 5.2</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>221.0 ± 32.0b</td>
<td>401.0 ± 23.0</td>
<td>245.0 ± 46.0b</td>
<td>432.0 ± 27.0</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>238.0 ± 7.0c</td>
<td>367.0 ± 48.0</td>
<td>245.0 ± 23.0c</td>
<td>343.0 ± 39.0</td>
</tr>
</tbody>
</table>

*ETBRd, endothelin B receptor deficient; STZ, streptozotocin; WT, wild type. Data are mean ± SD.

(P < 0.001). After induction of diabetes, there is a marked and significant rise of systolic BP to a maximum of 188 ± 12 mmHg in the ETBRd-STZ group, whereas BP falls slightly but significantly in the STZ group (Figure 1).

Kidney Function and Morphology

Kidney function deteriorates faster in the diabetic ETBRd animals. After 10 wk, plasma creatinine (53 ± 3.4 versus 39 ± 3.4 μmol/L; P < 0.01; Figure 2A) and albuminuria (10.4 ± 5.0 versus 2.3 ± 3.9 mg/d; P < 0.01; Figure 2B) are increased in the ETBRd-STZ group as compared with the STZ group. The same applies for the decrease of creatinine clearance (ETBRd-STZ 3.2 ± 0.8 versus STZ 4.3 ± 0.9 ml/min per kg; Table 2), whereas there is only a trend toward an increase of plasma urea (ETBRd-STZ 17 ± 3.9 versus STZ 13 ± 4.7 mmol/L; P = 0.09; Table 2). Morphologic data are summarized in Table 2. The media/lumen ratio of the intrarenal arteries is significantly elevated in the ETBRd-STZ group as compared with the STZ group (P < 0.01; Table 2). Changes of the media/lumen ratio of the intracardiac arteries follow the same pattern as in the kidney (Table 2). Representative micrographs showing significantly enhanced glomerulosclerosis in the diabetic groups are shown in Figure 3. There is a trend toward an increased glomerulosclerosis index in the ETBRd-STZ group as compared with the STZ group (P = 0.12).

BP-Regulating Systems

To identify mechanisms that are involved in the diabetes-induced hypertension in ETBRd rats, we further analyzed several candidate systems: The renin-angiotensin-aldosterone system (RAAS), the NO system, and the ET system. There is no evidence of the circulatory RAAS being upregulated in the diabetic ETBRd group. On the contrary, PRA, PRC, and urinary aldosterone excretion is significantly lower in the diabetic ETBRd group. On the contrary, PRA, PRC, and urinary aldosterone excretion is significantly lower in the diabetic ETBRd group. However, systematic evaluation of renin- and nNOS-positive glomeruli in the diabetic ETBRd group as compared with the STZ group (Table 3) is present as far as the immunohistochemical evaluation of renin- and nNOS-positive glomeruli is concerned. Evaluation of nNOS was performed because studies in the in vitro perfused juxtaglomerular apparatus give some evidence that nNOS is acting as a positive modulator of renin secretion (26). The quantity of the aldosterone-inducible α subunit of ENaC is not significantly different among the four groups in the Western blot analysis (data not shown). Electro-

Figure 1. Systolic BP over the course of the experiment. *P < 0.05, **P < 0.01 versus nondiabetic group of same genotype. Data are mean ± SD.

Figure 2. Plasma creatinine (A) and urinary albumin excretion (UAЕ) (B) over the course of the experiment. UAЕ of the streptozotocin-induced diabetic (STZ) group in week 10 is not yet significantly elevated compared with wild-type (WT) rats. However, UAЕ per kg body weight is significantly elevated in the STZ group (data not shown). *P < 0.05, **P < 0.01, ***P < 0.001 versus nondiabetic group of same genotype; †P < 0.05, ††P < 0.01 versus STZ; ‡P = 0.08 versus WT. Data are mean ± SD.

**Table 1. Plasma/urine glucose, osmolality, fluid balance, body weight, and heart rate (week 10 after induction of diabetes)**
lyte excretion is not significantly different between both diabetic groups (Table 3).

Because it is widely known that ET-1 via the ETBR activates endothelial NO synthase (eNOS), we analyzed urinary excretion of NO metabolites and its second messenger cGMP as overall markers of NO production. Nitrogen intake was similar in all groups as calculated from food intake (data not shown). Diabetic ETBRd rats show an elevated urinary nitrite/nitrate excretion (183%; \( P < 0.07 \); Table 3) and cGMP excretion (Figure 4).

The urinary ET-1 excretion (as an approximation of renal ET-1 synthesis) is strongly elevated in both diabetic groups at the end of the study (Table 3). Plasma ET-1 concentrations are significantly elevated in the ETBRd-STZ group as compared with the ETBRd group 5 wk after induction of diabetes, whereas there is no elevation in the STZ group at any time (Figure 5).

To quantify renal ET receptor expression, we performed a Western blot analysis. Figure 6 depicts the regulation of renal ET receptors. ETBR deficiency causes a moderate downregulation of renal ETAR, whereas diabetes leads to a 57% increase of renal ETBR.

**Discussion**

This study demonstrates for the first time that ETBRd diabetic rats that are on a normal-sodium diet develop severe low-renin hypertension and albuminuria and a mild reduction of creatinine clearance. The strong BP rise in the ETBRd-STZ rats seems not to be caused by activation of the circulating RAAS or by suppression of the NO system. The elevated plasma ET-1, possibly reflecting a reduced ETBR-dependent elimination of ET-1, seems to cause the severe hypertension via the remaining ETAR.

The STZ model in our setting reflects an early stage of diabetic nephropathy. The STZ-treated rats do not yet develop significant albuminuria and show only a modest rise of plasma creatinine and vascular kidney damage after 10 wk. In contrast, the simultaneous presence of ETBR deficiency and diabetes causes severe hypertension and enhanced functional renal impairment within this period. We therefore created a rat model of a more advanced stage of diabetic nephropathy than the pure STZ model. This is similar to other STZ models with concomitant BP elevation such as spontaneously hypertensive rats (27) and (even more pronounced) transgenic (mRen-2)27 rats (28). In contrast to those models, a further significant BP rise happens after the induction of diabetes in ETBRd rats, which enhances the kidney damage mainly during the course of the experiment.

There are two possible explanations for the finding of enhanced albuminuria in the ETBRd diabetic rats. On the one hand, the ETBRd group 5 wk after induction of diabetes, whereas there is no elevation in the STZ group at any time (Figure 5).

To quantify renal ET receptor expression, we performed a Western blot analysis. Figure 6 depicts the regulation of renal ET receptors. ETBR deficiency causes a moderate downregulation of renal ETAR, whereas diabetes leads to a 57% increase of renal ETBR.
hand, the severe hypertension on top of hyperglycemia certainly accelerates its occurrence. On the other hand, increased renal ET-1 activity (reflected by increased urinary ET-1 excretion in diabetic rats) and reduced clearance (because of ETBR deficiency) might contribute to its development. Renal ET-1 has been implicated in podocyte damage and development of proteinuria (29), and ET receptor antagonists are known to reduce proteinuria and renal matrix protein expression in rats with STZ-induced diabetes (31).

The decrease of kidney function and the BP rise in the ETBRd-STZ group go in parallel. Although we cannot rule out that the BP rise results secondary to the kidney damage, we assume the opposite, because the differences of renal impairment between both diabetic groups do not seem strong enough to explain the pronounced differences of BP.

Because recent literature about renin in diabetes is controversial, we used several independent methods to evaluate its possible involvement in BP regulation in our model. We could not detect changes in PRA, PRC, and renin-positive glomeruli in the STZ group after 10 wk. Reports of an elevated PRA (30) contrast with others reporting reduced PRA in patients with type 1 diabetes (31). A study with STZ-treated rats showed a reduction of PRA after 4 wk (32), whereas an older study observed a biphasic reaction with an increase in the first week followed by a reduction of PRA until week 8 (33). Our results show a highly significant reduction of circulating renin only in the ETBRd-STZ group. This downregulation supposedly can be attributed to a negative feedback mechanism as a result of severe hypertension. Our results do not rule out a diabetes-induced activation of local tissue RAAS, e.g., in the kidney or the heart. Conversely, urinary aldosterone excretion is elevated in both diabetic groups. However, aldosterone excretion in ETBRd rats is much lower than in the diabetic WT animals and:

### Table 3. Evaluation of possible mechanisms that lead to hypertension in the ETBRd-STZ group (week 10)*

<table>
<thead>
<tr>
<th></th>
<th>ETBRd-STZ</th>
<th>ETBRd</th>
<th>STZ</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma renin activity (ng/ml per h)</td>
<td>0.1 ± 0.01&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 1.7</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Plasma renin concentration (ng/ml per h)</td>
<td>0.5 ± 0.1&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>3.4 ± 0.9</td>
<td>9.1 ± 16.9</td>
<td>4.2 ± 1.7</td>
</tr>
<tr>
<td>Renin-positive sites/100 glomeruli</td>
<td>5 ± 3</td>
<td>15 ± 8</td>
<td>11 ± 5</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>nNOS-positive sites/100 glomeruli</td>
<td>63 ± 8</td>
<td>91 ± 24</td>
<td>84 ± 16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>126 ± 10</td>
</tr>
<tr>
<td>Aldosterone excretion (pmol/d)</td>
<td>19 ± 7&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>8 ± 5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>32 ± 52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Plasma sodium (mmol/L)</td>
<td>123.0 ± 3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>133.0 ± 2.1</td>
<td>126.0 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>134.0 ± 1.7</td>
</tr>
<tr>
<td>Urine sodium excretion (mmol/d)</td>
<td>0.5 ± 0.4</td>
<td>0.4 ± 0.3</td>
<td>0.2 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Urine potassium excretion (mmol/d)</td>
<td>1.6 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.8 ± 0.3</td>
<td>1.5 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Nitrite/nitrate excretion (μmol/d)</td>
<td>1.1 ± 0.8</td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Urine ET-1 excretion week 5 (pg/d)</td>
<td>116 ± 86</td>
<td>75 ± 69</td>
<td>204 ± 309</td>
<td>47 ± 49</td>
</tr>
<tr>
<td>Urine ET-1 excretion week 10 (pg/d)</td>
<td>723 ± 891&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55 ± 35</td>
<td>1108 ± 945&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36 ± 12</td>
</tr>
</tbody>
</table>

*Data are mean ± SD; n = 6 to 9 per group, except renin- and nNOS-positive glomeruli (n = 3 per group). ET-1, endothelin-1. nNOS, neuronal NO synthase.

<sup>a</sup><sup>b</sup><sup>c</sup><sup>d</sup><sup>e</sup><sup>f</sup><sup>g</sup><sup>h</sup>Significant differences versus nondiabetic group of same genotype.**Significant differences versus nondiabetic group of same genotype.††Significant differences versus WT.
therefore cannot explain the BP rise. It has been demonstrated in rats that ET-1 exerts a secretagogue effect on adrenal aldosterone secretion that seems to be mediated primarily by ETBR (34). The reduced aldosterone excretion in the ETBRd groups might be attributed to the lack of ETBR in the adrenal zona glomerulosa. We assume that (pseudo)hyponatremia, hyperosmolality, and increased urinary potassium excretion are due to diabetes-induced electrolyte disturbances and acidosis independent of the genotype (35).

Another important BP-regulating system is the NO-cGMP system. This is especially interesting because it is widely known that ET-1 via the ETBR activates eNOS (36). Our results suggest that the system is not downregulated and support the concept of a secondary counterregulation of the NO system. Excretion of NO end products and even more excretion of its second messenger cGMP is upregulated in ETBRd-STZ rats. This suggests that in our model, the NO system seems activated by mechanisms that do not involve the ETBR. However, because urinary nitrate/nitrite and cGMP concentrations give only a limited reflection of the endogenous NO system activity, definite conclusions cannot be drawn.

The mechanisms underlying the (reversible) bradycardia that typically is found in the STZ model are not fully understood (37). Studies in isolated cardiac preparations indicate that STZ-induced diabetes is associated with a depression in basal spontaneous pacemaker rate (38). However, chronotropic responsiveness to catecholamines was found to be intact (38) or even increased (39). Catecholamine action as a cause of hypertension therefore is unlikely as we would expect an associated rise of heart rate, which in contrast does not differ between both diabetic groups.

In the diabetic ETBRd rats, plasma ET-1 is strongly elevated. ET-1 is one of the most potent vasoconstrictors known and via the remaining ETAR possibly causes the severe hypertension. An exogenous two-fold increase of plasma ET-1 already causes a significant rise of systemic vasoconstriction and BP in vivo (40–42), whereas there is a six-fold increase of plasma ET-1 in this study. However, exogenous administration of ET-1 might not be directly comparable to conditions of this study. Different mechanisms might be involved in the rise of plasma ET-1 in ETBRd-STZ rats.

The ETBR acts as a clearance receptor, eliminating circulating ET-1 (43,44). Consistently, our study confirms elevated ET-1 plasma concentrations in the ETBRd group (+76% versus WT at study end) as described previously (15). The concomitant presence of diabetes causes an even stronger rise of circulating ET-1 (+503% versus WT at study end), which is not present in diabetic animals with intact ETBR. The observed upregulation of ETBR in diabetic animals might be necessary to eliminate the increased amount of ET-1 produced in diabetes (8,45). The rise of plasma ET-1 in ETBRd-STZ rats therefore might be attributed to the synergistically acting effects of diabetes-induced activation of the ET system and an impaired ability to eliminate the increased quantity of ET-1 via the ETBR.

The renal ET system is activated in diabetic animals as described previously (8,9). Urinary ET-1 excretion as an approximation of renal ET synthesis is equally elevated in both diabetic groups only at the end of the experiment. This indicates that the elevated plasma ET-1 is not caused by an enhanced diabetes-induced activation of the ET system and an impaired ability to eliminate the increased quantity of ET-1 via the ETBR.

Another, however speculative, hypothesis to explain our findings is that of an activated vascular ET system as the source of the increased plasma ET-1, because the endothelium is the most probable structure that is able to liberate large amounts of ET-1. A generalized endothelial dysfunction in patients with diabetes leading to the liberation of ET-1 has already been discussed (46), and the liberation of ET-1 in a state of endothelial dysfunction was observed previously (47). Moreover, ETBR seem to play a role in this context as they have been shown to be upregulated in vessels of diabetic rats (45). However, further studies are needed to clarify those issues.

Study Limitations and Outlook

In the diabetic ETBRd rats, BP, plasma ET-1, creatinine, and albuminuria rise in parallel. Because of the study design chosen to answer the initial hypothesis, it is difficult to reconcile what

Figure 6. Western blot for renal ETAR and ETBR; n = 3 per group. Band size is approximately 41 kD for ETAR and approximately 37 kD for ETBR. *P < 0.05 versus nondiabetic group of same genotype; †P < 0.05 versus STZ; ‡P < 0.05 versus WT. Data are mean ± SD.
occurs first: Hypertension and renal damage followed by ET-1 increases or ET-1 activation as a result of decreased clearance followed by BP rise and renal damage. For addressing the role of ET-1 in mediating the hemodynamic and structural changes, further study designs should consider two approaches. One is treating the hypertension with a non-ET modulator such as a vasodilator to determine whether this abolishes structural changes in the kidney. The second is to administer long term an ET antagonist to sort out the direct contribution of the ET-1/ETAR complex.

Conclusion
Our data do not support the initial hypothesis that a reduction of ETBR activity inhibits the progression of diabetic nephropathy. However, there might be differences between our ETBRd animal model and pharmacologic antagonism. This assumption is supported by the finding that diabetes does not affect BP in rats that are heterozygous for the ETBR defect (T.P. and B.H., unpublished observations), thus indicating that ETBR antagonists that block only part of the receptors (as do most available substances) might have different effects. However, BP and kidney function should be monitored closely if ETBR antagonistic drugs are administered to patients with diabetes in future studies.

Acknowledgment
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