Effects of endothelin or angiotensin II receptor blockade on diabetes in the transgenic (mRen-2)27 rat

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Background. Endothelin (ET) and angiotensin II (Ang II) are vasoactive/trophic peptides that may contribute to the progression of diabetic nephropathy. The transgenic (mRen-2)27 rat exhibits overexpression of Ang II at sites of normal physiological expression. Unlike other rat strains, the streptozotocin-induced diabetic Ren-2 rat develops progressive renal pathology associated with a declining glomerular filtration rate (GFR) and provides a convenient model to evaluate the role of these vasoactive peptides in the nephropathic process.

Methods and Results. Oral administration of either the endothelin A (ET A) and ET B receptor antagonist bosentan or the angiotensin type 1 (AT 1) receptor antagonist valsartan for 12 weeks reduced systolic blood pressure (SBP) of nondiabetic and diabetic Ren-2 rats to normotensive levels. Diabetic renal pathology was associated with intense renin mRNA and protein in the proximal tubules and juxtaglomerular cells along with overexpression of transforming growth factor-β1 (TGF-β1) and collagen IV mRNA in glomeruli and tubules. With valsartan but not bosentan, renin mRNA and protein in the proximal tubules were not detected. Valsartan but not bosentan reduced TGF-β1 and collagen IV mRNA and the severity of diabetic renal pathology. A declining GFR with diabetes was attenuated by both treatments. Albuminuria in diabetic rats rose further with bosentan but was reduced with valsartan.

Conclusions. Despite producing normotension, severe diabetic renal pathology was not prevented by bosentan, suggesting dissociation of ET, albuminuria, and hypertension from the structural injury in this diabetic model. The beneficial effects afforded by valsartan therapy strengthen the importance of the local renin-angiotensin system in mediating progressive diabetic renal injury.

The control of systemic and possibly intraglomerular hypertension is a key factor in the management of diabetic nephropathy [1]. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin type 1 receptor antagonists (AT 1) appear to be particularly effective in this regard in both human and experimental diabetic nephropathy [2–4]. Micropuncture studies have indicated that renoprotection with these agents is associated with reductions in intraglomerular pressure [5, 6]. More recently, the induction of proinflammatory and prosclerotic growth factors by hyperglycemia [7–10] and possibly tubular protein load [11] have also been recognized as important contributors to the microvascular and tubulointerstitial pathology of diabetic renal disease [12]. In various experimental models of renal disease, including diabetes, mRNA for transforming growth factor-β1 (TGF-β1) and collagen IV increases in the kidney with evidence that blockade of the renin-angiotensin system (RAS) decreases expression of these growth factors and matrix proteins as well as renal pathology [12, 13]. This functional interrelationship between angiotensin II (Ang II) and TGF-β may represent an important trophic/profihbrotic pathway involved in the progression of experimental diabetic nephropathy [14].

While the experimental streptozotocin (STZ)-induced diabetic rat develops proteinuria and modest expression of extracellular matrix (ECM), it is not associated with a loss of renal function. In contrast, the diabetic transgenic (mRen-2)27 rats develop severe renal impairment and histologic injury, which resembles human diabetic nephropathy. In the transgenic (mRen-2)27 rat, the mouse Ren-2 gene is inserted into the genome of a Sprague-Dawley rat such that overexpression of renin occurs at sites of normal physiological expression [15]. This model displays hypertension and develops progressive nephropathy with the induction of STZ diabetes, possibly because of the activation of the intrarenal RAS [16], and provides an opportunity to explore the relative importance of systolic blood pressure (SBP) versus the local RAS in the progression of diabetic nephropathy [16]. After an initial phase of hyperfiltration in the Ren-2
rat, STZ diabetes results in the rapid onset of renal impairment and severe renal pathology, with features similar to those observed in advanced human diabetic nephropathy. This progressive renal disease is prevented by ACE inhibitor treatment [16].

Endothelin (ET), a potent proinflammatory mediator, has also been implicated in the progression of various renal pathologies [17]. ET has been shown to be elevated in the diabetic kidney [18], and ET antagonists have been reported to reduce growth factor expression and ECM accumulation in glomeruli from diabetic rats [18, 19]. Furthermore, ET antagonists ameliorate renal pathology in several experimental models of kidney disease [20, 21]. In the kidney, ET-1 mRNA is reduced by ACE inhibition [19], and in vascular tissue, the effects of Ang II infusion are ameliorated by ET antagonism [22]. Together, these studies suggest a functional interrelationship between ET, Ang II, and structural injury.

Our current study compares an AT1 receptor antagonist with an ETA and ETB receptor antagonist at an equipotent antihypertensive dose to explore the relative involvement of Ang II and ET in mediating the renal functional and structural abnormalities observed in the STZ diabetic Ren-2 rat.

METHODS

Animals

Six-week-old female, heterozygous Ren-2 rats, weighing 125 ± 5 g, were randomized to receive either 55 mg/kg of STZ (Sigma, St. Louis, MO, USA) diluted in 0.1 mol/L citrate buffer, pH 4.5, or citrate buffer alone (nondiabetic) by tail vein injection following an overnight fast. Female rats were chosen because they do not require maintenance antihypertensive therapy. Diabetic and nondiabetic Ren-2 (N = 6 per group) were treated with an AT1 receptor antagonist, valsartan (average dose of 20 mg/kg/day, in drinking water; Novartis, Basel, Switzerland), or the ET (ETA and ETB) receptor antagonist bosentan (gavaged dose of 100 mg/kg/day; Roche, Basel, Switzerland) for 12 weeks post-STZ or vehicle. Treatment commenced within 24 hours of STZ injection. All rats were housed in a stable environment (maintained at 22 ± 1°C with a 12-h light-day cycle) and allowed free access to tap water and standard laboratory chow during this period. After 24 hours in metabolic cages, an aliquot of urine (5 mL) was collected from the 24-hour urine sample and stored at −70°C for subsequent analysis of urinary electrolytes and albumin concentration. Urinary Na+ and K+ concentrations and plasma electrolytes were measured in duplicate using an automated IL 943 flame photometer (Instrumentation Laboratories, Milan, Italy).

Albuminuria was determined by a double antibody radioimmunoassay, as previously performed in our laboratories [23]. The glomerular filtration rate (GFR) was determined by injecting a single shot of 99Tc-DTPA into the tail vein and sampling the blood after 43 minutes, as previously described [24].

Plasma preparation

Prior to sacrifice, plasma samples (100 µL) were collected in heparinized vials (10 U/mL) from the tail vein of conscious rats. The blood samples were centrifuged at 2000 × g for five minutes at 4°C, and the plasma supernatant was removed and frozen for later estimations of Na+ and K+.

Anatomical studies

Rats were anesthetized (Nembutal 60 mg/kg body wt intraperitoneally; Rhone Merieux, Pinkenba, QLD, Australia), and the abdominal aorta was cannulated with an 18-gauge needle. Perfusion-exsanguination commenced at SBP (180 to 220 mm Hg) via the abdominal aorta with 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4 (20 to 50 mL), to remove circulating blood, and the inferior vena cava adjacent to the renal vein was simultaneously severed, allowing free flow of the perfusate. After clearance of circulating blood, 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, was perfused for a further five minutes (100 to 200 mL of fixative) to fix the tissues [25]. Kidneys were removed from the animal, decapsulated, and sliced transversely. Kidneys were postfixed in the same fixative for two hours, routinely processed, embedded in paraffin, and sectioned.
Histopathology

Changes in kidney structure were assessed in a blinded protocol in at least 25 randomly selected tissue sections from each group studied. Sections were stained with either Mayer’s hematoxylin and eosin (HE) to examine cell structure, periodic acid-Schiff stain (PAS) to identify changes in basement membrane architecture and glycogen deposition, or Masson’s modified trichrome to demonstrate collagen matrix [26].

Glomerulosclerotic index

From 3 \( \mu m \) kidney sections stained with PAS, 150 to 200 glomeruli from rats were examined in a masked protocol. The degree of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4 by the method of Saito et al: grade 0, normal; grade 1, sclerotic area up to 25% (minimal); grade 2, sclerotic area 25 to 50% (moderate); grade 3, sclerotic area 50 to 75% (moderate to severe); and grade 4, sclerotic area 75 to 100% (severe) [27]. Glomerulosclerosis was defined as glomerular basement membrane (GBM) thickening, mesangial hypertrophy, and capillary occlusion. A glomerulosclerotic index (GSI) was then calculated using the formula:

\[
GSI = \frac{4}{\sum_{i=0}^{4} Fi (i)}
\]

where Fi is the percentage of glomeruli in the rat with a given score (i).

Immunohistochemistry

Three micron sections were placed into histosol to remove the paraffin wax, hydrated in graded ethanol, and immersed into tap water before being incubated for 20 minutes with normal goat serum (NGS) diluted 1:10 with 0.1 mol/L PBS, pH 7.4. Sections were then incubated for 18 hours at 4°C with specific primary antisera to renin protein. Sections incubated with 1:10 NGS instead of the primary antiserum served as the negative control. After thorough washing with PBS (3 \( \times 5 \) min changes), the sections were flooded with a solution of 5% hydrogen peroxide, rinsed with PBS (2 \( \times 5 \) min), and incubated with biotinylated goat antirabbit IgG (Dakopatts, Glostrup, Denmark) diluted 1:200 with PBS. Sections were rinsed with PBS (2 \( \times 5 \) min) and incubated with an avidin-biotin peroxidase complex (Vector, Burlingame, CA, USA) diluted 1:200 with PBS. Following rinsing with PBS (2 \( \times 5 \) min), sections were incubated with 0.05% diaminobenzidine and 0.05% hydrogen peroxide (Pierce, Rockford, IL, USA) in PBS, pH 7.6, for one to three minutes, rinsed in tap water for five minutes, counterstained in Mayer’s hematoxylin, differentiated in Scott’s tap water, dehydrated, cleared, and mounted in Depex [28].

The nature and specificities of the antisera raised to mouse and rat renin protein have been previously published by our laboratory [25, 29]. The polyclonal mouse renin protein antibody was raised against pure mouse submandibular gland renin [25], and the polyclonal rat renin protein was raised in rabbits against a fusion protein that was incorporated into Escherichia coli [29]. These antisera specifically label juxtaglomerular cells of the kidney, zona glomerulosa cells of the adrenal, and macroglial Müller cells of the retina. Renin protein antisera reveal the presence of both prorenin and partially or fully processed active renin.

In situ hybridization

The 1.4 kb cDNA probe for rat renin (gift of Dr. D.J. Campbell, Melbourne, Australia) was cloned into pGEM 4 (Stratagene, La Jolla, CA, USA) and linearized with Bam HI to produce an antisense riboprobe with T7 RNA polymerase. The 945 bp cDNA probe for TGF-\( \beta 1 \) (gift of Dr. Qian, Bethesda, MD, USA) and the 600 bp cDNA probe for \( \alpha 1 \) (IV) collagen (gift of Dr. R. Timpl, Martinsried, Germany) were also cloned into pBluescript KS+ (Stratagene) and linearized with XbaI and HindIII to produce antisense riboprobes with T7 RNA polymerase. In situ hybridization was performed on 4 \( \mu m \) paraffin sections, which were hybridized with antisense probes to renin, TGF-\( \beta 1 \), and collagen IV [12]. Tissue sections were dewaxed in histosol, hydrated through graded ethanol, and immersed in distilled water. Sections were then washed in 0.1 mol/L PBS, pH 7.4, and hybridized with \( ^{3}P \)-labeled antisense and sense-specific probes (5 \( \times 10^6 \) cpm/25 \( \mu L \) hybridization buffer), which were added to hybridization buffer (300 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5, 10 mmol/L Na2HPO4, pH 6.8, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0, 1 \( \times \) Denhardt’s solution, 0.8 mg yeast RNA/mL, 50% deionized formamide, and 10% dextran sulfate), heated to 85°C and 25 \( \mu L \) added to the sections. Cover slips were placed on the sections, and the slides were incubated in a humidified chamber (50% formamide) at 60°C for 14 to 16 hours. Slides were then washed in 2 \( \times \) standard saline citrate (SSC; 0.3 mol/L NaCl, 0.33 mol/L Na2C2H3O2) containing 50% formamide at 50°C to remove the cover slips. The slides were again washed with 2 \( \times \) SSC, 50% formamide for a further one hour at 55°C. Sections were then rinsed three times in RNase buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, pH 8.0, 0.5 mol/L NaCl) at 37°C and treated with 150 g RNase A/mL in RNase buffer for a further one hour at 37°C and then washed with 2 \( \times \) SSC at 55°C for 45 minutes. Finally, sections were dehydrated through graded ethanol, air dried, and exposed to Kodak X-Omat Autoradiography film for four days at room temperature. Slides were coated with Ilford K5 emulsion (Ilford, 1:1 with distilled water), stored with desiccant at room temperature for 21 days, developed in Kodak D19, fixed in...
Ilford Hypam, and stained with HE. For quantification of in situ hybridization, images of kidney sections were captured and digitized using a Fujix HC-2000 digital camera (Fuji, Tokyo, Japan). Regional gene expression was quantitatively measured using an image analysis system (AIS Imaging, Ontario, Canada), whereby the proportional area occupied by autoradiographic grains was measured against the total area of section from a minimum of three sections per animal (N = 6 animals per group), as previously described [30].

Statistics
Data are expressed as means ± SEM unless otherwise stated. Statistical significance was determined by a two-way analysis of variance (ANOVA) with a Fishers post hoc comparison. Albuminuria was analyzed using log transformed data and a represented as geometric means ×/× tolerance factors. Analyses were performed using Statview II + Graphics package (Abacus Concepts, Berkeley, CA, USA) on an Apple Macintosh G3 computer (Apple Computer, Inc., Cupertino, CA, USA). A P value < 0.05 was regarded as statistically significant.

RESULTS
Renal functional and biochemical studies

Body weight, systolic blood pressure and plasma glucose. Diabetic rats had reduced body weight independent of treatment (P < 0.01). SBP was decreased to a similar level within one week of commencement of either valsartan or bosentan in diabetic rats (P < 0.01; Table 1). Plasma glucose was elevated to a similar extent in all diabetic rat groups, independent of treatment (P < 0.01; Table 1).

Plasma and urinary electrolytes. In drug-treated diabetic rats, plasma K+ was increased compared with drug-treated nondiabetic controls (P < 0.05). Urinary electrolyte excretion was increased in diabetic rats, irrespective of drug treatment. Valsartan-treated diabetic rats displayed the largest increase in urinary electrolyte output (P < 0.05; Table 1).

Renal parameters. Diabetes was associated with an increase in urinary albumin excretion. Bosentan treatment increased albumin excretion further in both nondiabetic and diabetic rats, while valsartan treatment was associated with reduced albumin excretion in diabetic rats (Table 2). The impairment in GFR observed in diabetic Ren-2 rats was attenuated by all treatment regimens, although the GFR was still significantly lower than the respective drug-treated nondiabetic rats (Table 2).

Anatomical studies
In the kidney cortex of nondiabetic rats, there was no significant renal pathology (Fig. 1A). However, in diabetic rats, most glomeruli displayed thickened GBM, mesangial expansion, and capillary occlusion, as well as efferent arteriole hyalinization and tubular vacuolation (Fig. 1B). In both nondiabetic and diabetic rats treated with valsartan (Fig. 1 C, D) and nondiabetic rats treated with bosentan (Fig. 1E), only minimal evidence of GBM thickening was apparent. Diabetic rats treated with bosentan (Fig. 1F) displayed severe glomerulosclerosis and tubulointerstitial disease.

In the medulla of nondiabetic and diabetic rats treated

Table 1. Body weight, systolic blood pressure, and plasma glucose of nondiabetic and diabetic transgenic (mRen-2)27 treated for 12 weeks with either valsartan or bosentan

<table>
<thead>
<tr>
<th></th>
<th>Body weight</th>
<th>Systolic BP</th>
<th>Glucose</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Urine μmol/min</th>
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<tr>
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<td>g</td>
<td>mm Hg</td>
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<td></td>
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<tr>
<td>Nondiabetic</td>
<td>291 ± 9</td>
<td>219 ± 9</td>
<td>5.2 ± 0.3</td>
<td>140 ± 3</td>
<td>4.4 ± 0.2</td>
<td>1.31 ± 0.3</td>
</tr>
<tr>
<td>Nondiabetic + V</td>
<td>301 ± 7</td>
<td>133 ± 6</td>
<td>6.3 ± 0.5</td>
<td>141 ± 3</td>
<td>4.0 ± 0.2</td>
<td>1.04 ± 0.2</td>
</tr>
<tr>
<td>Nondiabetic + B</td>
<td>332 ± 7</td>
<td>139 ± 4</td>
<td>5.9 ± 0.6</td>
<td>138 ± 4</td>
<td>4.0 ± 0.2</td>
<td>1.18 ± 0.1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>218 ± 8b</td>
<td>231 ± 6</td>
<td>21.7 ± 0.9</td>
<td>143 ± 2</td>
<td>6.8 ± 0.3c</td>
<td>3.30 ± 0.5c</td>
</tr>
<tr>
<td>Diabetic + V</td>
<td>243 ± 14a</td>
<td>134 ± 7</td>
<td>21.4 ± 0.7</td>
<td>140 ± 4</td>
<td>4.7 ± 0.2</td>
<td>2.61 ± 0.57a</td>
</tr>
<tr>
<td>Diabetic + B</td>
<td>249 ± 14b</td>
<td>129 ± 7</td>
<td>19.6 ± 0.6</td>
<td>141 ± 4</td>
<td>5.4 ± 0.2</td>
<td>3.76 ± 0.17</td>
</tr>
</tbody>
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Values are expressed as means ± SEM; N = 6 rats per group. Abbreviations are: V, valsartan; B, bosentan. 

Table 2. Albuminuria and glomerular filtration rate in nondiabetic and diabetic transgenic (mRen-2)27 rats treated for 12 weeks with either valsartan or bosentan

<table>
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<tr>
<th></th>
<th>Albuminuria mg/24 h</th>
<th>GFR mL/min</th>
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<tr>
<td>Nondiabetic</td>
<td>0.30 ± 0.10</td>
<td>3.35 ± 0.1</td>
</tr>
<tr>
<td>Nondiabetic + V</td>
<td>0.42 ± 0.08b</td>
<td>3.21 ± 0.1</td>
</tr>
<tr>
<td>Nondiabetic + B</td>
<td>2.90 ± 0.88c</td>
<td>3.64 ± 0.2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>2.42 ± 0.20c</td>
<td>1.56 ± 0.1c</td>
</tr>
<tr>
<td>Diabetic + V</td>
<td>1.20 ± 0.10b</td>
<td>2.45 ± 0.14b</td>
</tr>
<tr>
<td>Diabetic + B</td>
<td>6.17 ± 0.40b</td>
<td>2.32 ± 0.12b</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM; N = 6 rats per group. V, valsartan; B, bosentan; GFR, glomerular filtration rate. 

*P < 0.05 when compared with respective nondiabetic rats

*P < 0.05 when compared to respective untreated rats
with valsartan (Fig. 2 C, D) and nondiabetic rats treated with bosentan (Fig. 2E), there was no evident pathology. However, in diabetic rats (Fig. 2B) and diabetic rats treated with bosentan (Fig. 2F), there was florid deposition of collagen matrix, tubular dilation and degeneration, and the presence of inflammatory cells.

**Renin mRNA and immunohistochemistry**

In nondiabetic rats, some juxtaglomerular apparatuses (JGAs) were positive for renin mRNA (Fig. 3A) or protein (Fig. 4A), whereas with diabetes, most JGAs and proximal tubules displayed intense labeling for renin mRNA (Fig. 3B) and protein (Fig. 4B). Nondiabetic rats treated with either valsartan or bosentan (data not shown) and diabetic rats treated with valsartan displayed labeling for renin mRNA (Fig. 3C) and protein (Fig. 4C) in JGA only. Diabetic rats treated with bosentan displayed intense labeling for renin mRNA (Fig. 3D) and protein (Fig. 4D) in both JGA and proximal tubules.

**Transforming growth factor-β1 and collagen IV mRNA**

In nondiabetic rats, TGF-β1 (Fig. 5 A, B) and collagen IV (Fig. 6 A, B) mRNA were barely detected in the kidney, while in diabetic rats, TGF-β1 (Fig. 5 C, D) and collagen IV (Fig. 6 C, D) mRNA were increased (Fig. 7) and associated with sclerotic glomeruli and regions of

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Fig. 1. Histopathology of the kidney cortex in non-diabetic and diabetic transgenic (mRen-2)27 treated for 12 weeks with either valsartan or bosentan. Sections are stained with PAS. G, glomerulus. (A) In nondiabetic rats, there is minimal evidence of glomerulosclerosis. (B) Diabetic rats have severe glomerulosclerosis, arteriolar hyalinization (*). Valsartan-treated nondiabetic (C) and diabetic rats (D) and bosentan-treated nondiabetic rats (E) have minimal evidence of glomerulosclerosis. Bosentan-treated diabetic rats (F) have severe glomerulosclerosis and cortical interstitial fibrosis (arrow, magnification ×300). Reproduction of this figure in color was made possible by Novartis Pharmaceuticals, Bern, Switzerland.
Fig. 2. Histopathology of the kidney medulla in nondiabetic and diabetic transgenic (mRen-2)27 treated for 12 weeks with either valsartan or bosentan. Sections are stained with Masson’s Trichrome. VR, vasa recta. (A) Nondiabetic rats have no evidence of medullary pathology, whereas (B) diabetic rats have dilated tubules, degenerated vasa recta bundles, and the presence of inflammatory cells (arrow), and there appeared to be more collagen (stained blue). In valsartan-treated nondiabetic (C) and diabetic rats (D) and bosentan-treated nondiabetic rats (E), there is no evidence of medullary pathology. Bosentan-treated diabetic rats (F) have dilated tubules, degenerated vasa recta bundles and the presence of inflammatory cells (arrow), and there appears to be more collagen (stained blue) throughout the interstitium (magnification ×300). Reproduction of this figure in color was made possible by Novartis Pharmaceuticals, Bern, Switzerland.

DISCUSSION

Blockade of the RAS with AT1 antagonists or ACE inhibitors has been shown previously to improve glomerular hemodynamics and structure in human and rodent diabetic nephropathy [2, 4, 5, 31, 32]. The present findings provide further evidence for a direct causal relationship between the activity of the intrarenal RAS and the pro-tubulointerstitial disease. In valsartan-treated nondiabetic (not shown) and diabetic rats, the levels of TGF-β1 (Fig. 5 E, F) and collagen IV (Fig. 6 E, F) mRNA were similar to nondiabetic rats (Fig. 7). In bosentan-treated nondiabetic rats, both TGF-β1 and collagen IV mRNA (data not shown) were similar to nondiabetic rats (Fig. 7). In diabetic rats treated with bosentan, TGF-β1 (Fig. 5 G, H), and collagen IV (Fig. 6 G, H), mRNA was localized to glomeruli and areas of tubulointerstitial disease, similar to that seen in diabetic rats (Fig. 7).

Glomerulosclerotic index

Diabetes was associated with an increase in the GSI. The GSI in diabetic rats treated with valsartan was significantly lower than in diabetic rats (P < 0.05; Table 3). The GSI in diabetic rats treated with bosentan was not significantly reduced when compared with diabetic rats (Table 3), but there were fewer severely sclerotic glomeruli.
Fig. 3. Renin mRNA of the kidney in nondiabetic and diabetic transgenic (mRen-2)77 treated for 12 weeks with either valsartan or bosentan. Sections are stained with HE. (A) Nondiabetic rats displayed renin mRNA (arrow) in the JGA, whereas in (B) rats with diabetes there was intense renin mRNA (arrow) in both the JGA and the proximal tubules. In diabetic rats treated with valsartan (C), renin mRNA (arrow) was detected in the JGA only. In diabetic rats treated with bosentan (D), gene expression for renin mRNA (arrow) was present in both JGA and the cytoplasm of proximal tubules (magnification ×300). Reproduction of this figure in color was made possible by Novartis Pharmaceuticals, Bern, Switzerland.

gression of diabetic nephropathy. As with ACE inhibition in our previous study [16], treatment with the AT1 antagonist valsartan reduced SBP, ameliorated the severe pathology, and improved the renal function of diabetic Ren-2 rats (Table 4). By contrast, bosentan, although normalizing blood pressure and improving GFR, had little or no effect on the florid renal pathology and was associated with increased albuminuria (Table 4).

Bosentan reduced SBP to normotensive levels in both nondiabetic and diabetic Ren-2 rats, suggesting that the hypertension of this Ren-2 strain is ET dependent. These findings are in accordance with other investigators who have shown that the hypertension in the Ren-2 rat is probably mediated by locally generated Ang II and ET [33]. This is in accordance with experimental data, which have shown that the chronic pressor properties of infused Ang II in normal rats are blunted by bosentan [34]. In the present study, hypertension in the Ren-2 rat appeared to be partly ET dependent, yet blockade of the ET receptor failed to attenuate the diabetic renal pathology. Therefore, it appears that the vasoconstrictor effects of hormones such as ET and Ang II can be functionally separated from their actions in the microvascular tissues. Indeed, agents such as AT1 antagonists or ACE inhibitors can confer renoprotective benefits without major effects of systemic blood pressure, as has been observed in studies performed in normotensive diabetic rats [3]. Despite only a modest reduction in SBP, agents that interrupt the RAS confer renoprotection in diabetes [5].

The finding that bosentan did not reduce renal pathol-
Fig. 4. Renin immunohistochemistry of the kidney in nondiabetic and diabetic transgenic (mRen-2)27 treated for 12 weeks with either valsartan or bosentan. Sections are counterstained with Mayer’s hematoxylin. G, glomerulus. (A) Nondiabetic rats displayed renin protein (arrow) in the JGA, whereas with (B) diabetic animals there was intense renin protein (arrow) in both the JGA and the proximal tubules. (C) In diabetic rats treated with valsartan, renin protein (arrow) was detected in the JGA only. (D) In diabetic rats treated with bosentan, renin protein (arrow) was detected in both the JGA and the cytoplasm of proximal tubules (magnification ×300).

ogy in the diabetic Ren-2 contrasts with other reports of both hemodynamic and pathological benefits with the ET blockade in various forms of renal disease [20, 21]. In other models of experimental diabetes, treatment with another nonselective ET antagonist has reduced both albuminuria and SBP [21], whereas in the present study, ET antagonism decreased SBP but increased albuminuria. One must be cautious when comparing the two studies since the present studies were performed in transgenic rats that develop renal failure. In the diabetic Ren-2 rat, it is possible that the disease is predominantly Ang II dependent, although kidney and plasma Ang II levels actually decrease with diabetes [35]. The results of the present study suggest that local cell-specific autocrine/paracrine mechanisms may be more relevant than whole tissue peptide levels. Indeed, in the present study, there was evidence of a local increase in renin in proximal tubules of diabetic rat kidneys consistent with specific activation of the RAS within the kidney.

Although bosentan did not improve the overall GSI, there were significantly fewer severely sclerotic (grades 3 and 4) glomeruli when compared with untreated rats. This may explain the improvement in GFR in diabetic Ren-2 rats treated with bosentan. A discrepancy between the effects of ET receptor antagonism on glomerular function and structural injury has also been reported in a model of cyclosporine nephrotoxicity [36]. Other investigators have shown a correlation between GFR and occluded glomeruli in diabetes [37, 38]. This improvement in renal function could relate to the known beneficial effects of bosentan on glomerular and systemic hemodynamics [39]. ET-1 has been reported to increase intraglomerular pressure with ET blockade lowering glomerular pressure [40]; however, this is not a universal finding, as a study found that ET can reduce intraglomerular pressure, albeit at high doses [41]. ET-1 induces mesangial cell contraction [42], and therefore, ET blockade would be expected to increase the glomerular filtration surface area through mesangial cell relaxation. This effect on the glomerular barrier may explain the increased albuminuria associated with bosentan in the Ren-2 rats, irrespective of the presence of diabetes. Since albumin occurs as a result not only of the glomerulus but also tubular factors, one cannot exclude the effects
Fig. 5. Transforming growth factor-β1 mRNA in the kidney in nondiabetic and diabetic transgenic (mRen-2)27 treated for 12 weeks with either valsartan or bosentan. Sections were stained with HE. (A, C, E, and G) Bright-field micrographs. (B, D, F, and H) Corresponding dark-field micrographs. In both nondiabetic (A and B) and diabetic (E and F) rats treated with valsartan, there was minimal gene expression for TGF-β1 detected. However, in both diabetic (C and D) and diabetic rats treated with bosentan (G and H), there was intense gene expression for TGF-β1 in the glomerulus (G) and regions of tubulointerstitial injury (magnification ×300).
Fig. 6. Collagen IV mRNA in the kidney in nondiabetic and diabetic transgenic (mRen-2)27 treated for 12 weeks with either valsartan or bosentan. Sections were stained with HE. (A, C, E, and G) Bright-field micrographs. (B, D, F, and H) Corresponding dark-field micrographs. In both nondiabetic (A and B) and diabetic rats treated with valsartan (E and F), there was minimal gene expression for collagen IV detected. However, in both diabetic (C and D) and diabetic rats treated with bosentan (G and H), there was intense gene expression for collagen IV in the glomerulus (G) and regions of tubulointerstitial injury (magnification ×600).
Fig. 7. Quantitation of gene expression for TGF-β1 (A) and collagen IV (B). Values are expressed as means ± SEM (N = 6). ∗P < 0.05 when compared with respective nondiabetic rats; #P < 0.05 when compared with untreated diabetic rats.

Table 3. Glomerulosclerotic index and the percentage of glomerulosclerosis in kidneys from nondiabetic and diabetic transgenic (mRen-2)27 rats treated for 12 weeks with either valsartan or bosentan

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<tr>
<td>Nondiabetic</td>
<td>Grade 0</td>
</tr>
<tr>
<td></td>
<td>0.80 ± 0.10</td>
</tr>
<tr>
<td>Nondiabetic + V</td>
<td>0.24 ± 0.06b</td>
</tr>
<tr>
<td>Nondiabetic + B</td>
<td>0.42 ± 0.18</td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.75 ± 0.10a</td>
</tr>
<tr>
<td>Diabetic + V</td>
<td>1.07 ± 0.21b</td>
</tr>
<tr>
<td>Diabetic + B</td>
<td>1.60 ± 0.3a</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM; N = 6 rats per group.

*p < 0.05 when compared with respective nondiabetic rats

Table 4. Summary of results

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic (V)</th>
<th>Diabetic (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP</td>
<td>→</td>
<td>→</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>Albuminuria</td>
<td>→</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Serum K</td>
<td>→</td>
<td>↓</td>
<td>→</td>
<td>↑</td>
</tr>
<tr>
<td>GFR</td>
<td>→</td>
<td>↓</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Histopathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>→</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Medulla</td>
<td>→</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>Renin mRNA</td>
<td>→</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>JGA</td>
<td></td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>PCT</td>
<td></td>
<td>↓</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>Renin protein</td>
<td>→</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>JGA</td>
<td></td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>PCT</td>
<td></td>
<td>↓</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>TGF-β1 mRNA</td>
<td>→</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen IV mRNA</td>
<td>→</td>
<td>↓</td>
<td>→</td>
<td>→</td>
</tr>
</tbody>
</table>

Abbreviations are: V, valsartan; B, bosentan; GFR, glomerular filtration rate; SBP, systolic blood pressure; JGA, juxtaglomerular cells; PCT, proximal tubules; ND, not detected.
of ET antagonists on tubular reabsorption of albumin, an area not previously investigated in detail.

We have previously hypothesized from findings with the ACE inhibitor perindopril in this model that the appearance of renin in proximal tubules represents a potential pathogenic mechanism for tubulointerstitial disease [16]. As with perindopril, valsartan treatment of diabetic Ren-2 prevented the appearance of renin in proximal tubular cells and the progression of surrounding fibrosis and inflammation. On the other hand, bosentan neither prevented the appearance of tubular renin nor the development of interstitial disease. The appearance of tubular renin appears to indicate activation of a normally quiescent cellular RAS, which may then precipitate a cascade of Ang II-dependent proclerotic and proinflammatory growth factors such as TGF-β [13]. Evidence of synthesis of components of the local RAS in the tubule is confirmed by the presence of increased renin mRNA within the tubules of untreated diabetic and bosentan-treated diabetic Ren-2 rats.

Increased glomerular and tubulointerstitial expression of TGF-β1 and collagen IV mRNA in the diabetic and bosentan-treated diabetic Ren-2 rats may represent a pathogenic mechanism that involves amplification of the glomerular and tubular RAS with subsequent growth factor induction, resulting in the development of renal pathology. Elevated glucose also enhances the sensitivity of Ang II in the kidney [9], as well as increasing TGF-β and ECM [43]. Studies in a proximal tubule cell line have suggested that glucose per se may, in certain contexts, itself activate the RAS [44], providing a further mechanism for a diabetic induced amplification of Ang II-mediated tissue injury. Elevated glucose and local Ang II may then act synergistically to increase TGF-β1 and collagen IV, thereby accelerating renal damage further.

In summary, this study has reported a difference in the renal effects between AT1 and ETA receptor blockade in this model of progressive diabetic nephropathy, despite equivalent reduction in blood pressure. These findings highlight a dissociation of hypertension from diabetic renal disease and provide strong evidence for intrarenal RAS, particularly in the proximal tubules, to mediate tissue injury in the diabetic Ren-2 rat. This would ultimately result in the glomerular and tubulointerstitial disease observed in this model, possibly via the proclerotic cytokine TGF-β1. This leads to ECM accumulation, glomerulosclerosis, tubulointerstitial disease, and renal failure.

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