Tranilast Prevents the Progression of Experimental Diabetic Nephropathy through Suppression of Enhanced Extracellular Matrix Gene Expression

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ABSTRACT

The present study was performed to investigate the effects of the antiallergic drug tranilast on the development of diabetic nephropathy in streptozotocin (50 mg/kg)-induced diabetic spontaneously hypertensive rats (SHR). Diabetic SHR were given standard chow or chow containing tranilast at a dose of 1400 mg/kg for 24 weeks. The effects of tranilast on urinary albumin excretion, mesangial expansion, expression of transforming growth factor-β (TGF-β) and type I collagen mRNAs, number of anionic sites on the glomerular basement membrane (GBM), and urinary TGF-β and 8-hydroxy-2′-deoxyguanosine (8-OHdG) excretion were assessed. Tranilast did not affect the blood glucose concentration or blood pressure in diabetic SHR. Urinary albumin excretion rate and creatinine clearance were markedly increased in diabetic SHR. Tranilast treatment decreased albuminuria and hyperfiltration. Tranilast inhibited the diabetes-induced expansion of mesangial and tuft areas, as well as the increase in urinary TGF-β and 8-OHdG excretion, loss of anionic sites of GBM, and overexpression of TGF-β as determined immunohistochemically. The levels of TGF-β and type I collagen mRNA expression were increased in the renal cortex in untreated diabetic SHR at 24 weeks, as determined by real-time quantitative polymerase chain reaction. Tranilast treatment inhibited the up-regulation of TGF-β and type I collagen mRNA expression by 65 and 36%, respectively, in diabetic SHR. In conclusion, tranilast decreased albuminuria by suppressing glomerular hyperfiltration, mesangial expansion, and loss of the charge barrier via regulation of extracellular matrix gene expression and oxidative stress. Tranilast may be clinically useful in the treatment of diabetic nephropathy.

Diabetic nephropathy is a serious complication in diabetic subjects and is a leading cause of end-stage renal disease and mortality. The characteristic morphological and ultrastructural changes in patients with diabetic nephropathy are expansion of the mesangial matrix and loss of the charge barrier on the glomerular basement membrane (GBM). The presence of microalbuminuria in diabetic subjects predicts an increased risk for clinical proteinuria (Mogensen, 1984). It is important for subjects with microalbuminuria to obtain appropriate treatment as soon as they are identified (Alzaid, 1996). Hypertension frequently coexists in patients with type 2 diabetes and may be another risk factor accelerating cardiovascular and renal complications (UK Prospective Diabetes Study Group, 1998). Despite the beneficial effects of good glycemic control and antihypertensive therapy, diabetic nephropathy continues to progress in a significant proportion of patients, and therefore additional therapies are needed. Tranilast, \( N-(3',4'-\text{dimethoxyxycinnamoyl})-\text{anthranilic acid} \), is an antiallergic drug that has been used clinically, because it inhibits the release of both chemical mediators from mast cells and cytokines from macrophages (Suzawa et al., 1992). Tranilast has been reported to inhibit transforming growth factor-β (TGF-β) and to reduce collagen synthesis in experimental studies (Miyazawa et al., 1995; Ikeda et al., 1996; Bonnet et al., 2003). In fact, tranilast is now used clinically for the treatment of hypertrophic scars, scleroderma, and skin disorders associated with an excessive fibrotic response (Yamada et al., 1994; Shigeki et al., 1997). Tranilast has also been reported to reduce the restenosis rate after percutaneous transluminal coronary angioplasty (Tamai et al., 1999). Recently, tranilast was shown to attenuate tubulointerstitial...

**ABBREVIATIONS:** GBM, glomerular basement membrane; TGF-β, transforming growth factor-β; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; SHR, spontaneously hypertensive rat(s); SBP, systolic blood pressure; Cr, creatinine; Ccr, creatinine clearance; LRE, lamina rara externa; PCR, polymerase chain reaction; ECM, extracellular matrix; PKC, protein kinase C; AGE, advanced glycation end product.
fibrosis and albuminuria in a rat model of advanced experimental diabetic nephropathy (Mifsud et al., 2003). However, its effects on the renal pathology and actions of TGF-β in diabetic nephropathy in vivo have not been determined in detail. In the present study, we investigated whether tranilast can prevent the development of diabetic nephropathy and/or hypertensive nephrosclerosis by suppressing mesangial expansion and the overexpression of TGF-β in a hypertensive model of diabetic nephropathy.

Materials and Methods

Materials. Male spontaneously hypertensive rats were purchased from Charles River Japan (Tokyo, Japan). Chow was purchased from Oriental Yeast (Tokyo, Japan). Streptozocin (STZ), 2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose, was purchased from Sigma-Aldrich (St. Louis, MO). Tranilast was donated by Kissei Pharmaceutical Co. (Nagano, Japan). Enzyme-linked immunosorbent assay for the determination of albumin (Nephrat) was purchased from Sigma-Aldrich (St. Louis, MO). The monoclonal antibody for TGF-β1 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Envision kit was purchased from DakoCytomation (Kyoto, Japan). 3′,3′-Diaminobenzidine was purchased from Sigma-Aldrich. Rabbit anti-rat TGF-β antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Male spontaneously hypertensive rats, weighing 150 to 200 g and aged 10 to 12 weeks, were given a single intravenous injection of STZ (50 mg/kg) to induce diabetes, as described previously (Yamashita et al., 2002; Ota et al., 2003). The blood glucose concentration of all diabetic rats examined 72 h after injection was more than 16.7 mM before the experiments. After induction of diabetes, the diabetic SHR were divided into the following two groups: 1) rats given normal chow (diabetic, n = 10) and 2) rats given chow mixed with tranilast at a dose of 1400 mg/kg (diabetic + tranilast, n = 6). Nondiabetic SHR treated with or without tranilast were used as controls. Two groups of nondiabetic SHR were also investigated: 3) rats given normal chow (nondiabetic, n = 10) and 4) rats given chow mixed with tranilast at a dose of 1400 mg/kg (nondiabetic + tranilast, n = 6). The rats were allowed unrestricted access to water and to the standard or mixed chow. All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at the Takarumachi campus of Kanazawa University.

Blood Pressure Measurement. Systolic blood pressure (SBP) was assessed by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) in conscious pretrained rats before and 4, 8, 16, and 24 weeks after the start of tranilast treatment.

Blood Sampling and Analysis. Blood samples were obtained from the tail vein before and 4, 8, 16, and 24 weeks after the start of treatment. Blood glucose was measured by the glucose-oxidase method using Glucocard (Aventis Pharma, Tokyo, Japan). The blood samples were centrifuged, and serum was frozen at −70°C for subsequent measurement of the serum creatinine (Cr) concentrations by quantitative colorimetric assay (BML, Tokyo, Japan).

Urinary Albumin Excretion. Urinary albumin excretion (milligrams per 24 h) was assessed before and 4, 8, 16, and 24 weeks after the start of treatment. Rats were placed in individual metabolic cages to collect urine over a period of 24 h. Urine samples were collected and frozen at −70°C for subsequent analysis of albumin and Cr concentrations. Urinary albumin concentrations were measured by enzyme-linked immunosorbent assay using an anti-rat albumin antibody (Nephrat).

Hyperfiltration and Mesangial Expansion. Urinary Cr concentrations were measured by a quantitative colorimetric assay (BML). Creatinine clearance (Ccr, milliliters per minute per kilogram) was calculated as urinary Cr urine volume serum Cr −1 body weight −1. After 24 weeks of tranilast administration, the animals were killed, and their kidneys were fixed in 10% buffered formalin and embedded in paraffin. Light microscopy was performed using periodic acid-Schiff-stained 6-μm serial sections containing 40 to 60 glomeruli. The glomerular cross-sectional area (Ag), mesangial area (Am), and tuft area (At) were measured in 15 glomerular profiles per rat using NIH Image 1.58 software, as described previously (Ota et al., 2003). The glomerular volume (Vg) was then calculated as Vg = β/k [Ag] 3/2, where β = 1.38 is the size distribution coefficient and k = 1.1 is the shape coefficient for glomeruli idealized as a sphere (Weibel, 1979). All measurements were performed in a blinded manner.

Urinary Excretion of TGF-β. Urinary excretion of TGF-β (nanograms per 24 h) was assessed before and 4, 8, 16, and 24 weeks after the start of treatment. Urine samples were collected and frozen at −70°C until analysis. Urinary TGF-β concentrations were measured by competitive enzyme-linked immunosorbent assay kit (TGF-β1 E_max immunoassay system) according to the manufacturer’s instructions. To activate the latent urinary TGF-β to the immunoreactive form, each 50 μl of urine sample was first acid activated by incubation with 1 μl of 1.0 N HCl for 15 min at room temperature and neutralized by 1 μl of 1.0 N NaOH. Urinary TGF-β was expressed as total amount excreted in 24 h.

Immunohistochemical Analysis of TGF-β Expression in Glomeruli. After 24 weeks of tranilast administration, the animals were killed, and their kidneys were rapidly removed. Their kidneys were immediately frozen in liquid nitrogen, and sections were placed on gelatin-coated glass slides. Kidney specimens were incubated with rabbit anti-TGF-β antibody for 1 h at room temperature. This was followed by application of the immunoperoxidase technique using an Envision kit. Peroxidase activity was identified by reaction with 3′,3′-diaminobenzidine. Each incubation was followed by three washes in phosphate-buffered saline. Positive TGF-β staining in the glomeruli and interstitium were observed at 400× magnification by two independent observers in a blinded manner.

Visualization of Anionic Sites on the Lamina Rara Externa. To investigate the effects of tranilast on the anionic charge of the GBM, we evaluated the number of anionic sites on the LRE of the GBM, as described previously (Yamashita et al., 2002; Ota et al., 2003). Anionic sites were identified as particles that showed intense staining with polyethyleneimine. The number of anionic sites per 1000 nm of LRE was calculated on electron micrographs (average of 10 random visual fields) in each rat at a final magnification of 50,000×. Anionic sites were counted by two observers who were blinded to the treatment group.

Urinary Excretion of 8-OHdG. 8-OHdG is one of the most common markers for oxidative DNA damage and oxidative stress in vivo. Urinary excretion of 8-OHdG (nanograms per 24 h) was assessed before and 4, 8, 16, and 24 weeks after the start of treatment. Urine samples were collected and frozen at −70°C until analysis. Urinary 8-OHdG concentrations were measured by competitive enzyme-linked immunosorbent assay kit (8-OHdG check) according to the manufacturer’s instructions. Urinary 8-OHdG was expressed as total amount excreted in 24 h per body weight (kilograms).

Analysis of TGF-β and Type I Collagen mRNAs. Total RNA was extracted from each renal cortex by the acid guanidinium isothiocyanate-phenol-chloroform method, followed by synthesis of cDNA as described previously (Ota et al., 2003). Real-time quantitative polymerase chain reaction (PCR) was performed for TGF-β1 and type I collagen using the ABI Prism 7700 sequence detection system (Applied Biosystems) as described previously (Ota et al., 2003; Takamura et al., 2004). To control for variations in the amount of RNA available for PCR in the different samples, the levels of gene expression of the target sequence were normalized in relation to that
Results

Metabolic and Biochemical Parameters. Body weight, SBP, and blood glucose concentration at each time point in each group are summarized in Table 1. Tranilast administration did not affect the health of the rats, including food intake and body weight. The mean body weights of the groups were similar at the time of STZ injection. At each time point after tranilast administration, the mean body weight was significantly lower in diabetic SHR groups (diabetic and diabetic + tranilast) than in the nondiabetic SHR group (nondiabetic and nondiabetic + tranilast; Table 1). The mean SBP values were similar in all groups at all time points. The blood glucose concentration did not differ among all groups at the time of STZ injection. STZ-injected SHR groups showed an increase in blood glucose concentration at 2 weeks after STZ injection, whereas no differences were observed among diabetic SHR groups. Tranilast did not affect the body weight, SBP, or blood glucose concentrations of the diabetic SHR groups (Table 1).

Albuminuria. Urinary albumin excretion is shown in Fig. 1. In untreated diabetic SHR, albuminuria increased gradually from 8 to 24 weeks after the onset of diabetes. The degree of albuminuria was significantly greater in the untreated diabetic SHR (diabetic) than in untreated diabetic SHR treated with tranilast (diabetic + tranilast; Table 1). The mean urinary albumin excretion concentration was significantly lower in rats given tranilast compared with untreated diabetic SHR.

Glomeral Hyperfiltration. Ccr increased gradually from 8 to 16 weeks in untreated diabetic SHR (Fig. 2). Ccr was higher in untreated diabetic SHR than in nondiabetic SHR after 8 weeks, suggesting that hyperfiltration contributes to the development of albuminuria in diabetic SHR. Ccr was lower after 8 weeks in rats given tranilast compared with untreated diabetic SHR. Among the morphological parameters examined, the glomerular tuft was increased significantly in untreated diabetic SHR (diabetic) at 24 weeks of treatment compared with the values in nondiabetic SHR (nondiabetic and nondiabetic + tranilast; Table 2). The glomerular volume was also significantly increased in untreated diabetic SHR. Tranilast treatment ameliorated the increases in both tuft area and glomerular volume by suppressing glomerular hyperfiltration.

Mesangial Expansion. Table 2 shows the morphological parameters for each group at 24 weeks of treatment. In untreated diabetic SHR (diabetic), the mesangial area was significantly increased compared with nondiabetic SHR (nondiabetic and nondiabetic + tranilast). The mesangial area was further increased in diabetic SHR treated with tranilast (diabetic + tranilast).

Table 1

<table>
<thead>
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<th>Group</th>
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<th>4</th>
<th>8</th>
<th>16</th>
<th>24</th>
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<tr>
<td>Body weight (g)</td>
<td>174.2 ± 1.8</td>
<td>281.6 ± 2.1</td>
<td>335.4 ± 4.0</td>
<td>385.8 ± 1.6</td>
<td>410.0 ± 4.2</td>
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<td>Nondiabetic</td>
<td>171.7 ± 2.5</td>
<td>278.3 ± 2.6</td>
<td>346.5 ± 20.5</td>
<td>376.7 ± 3.1</td>
<td>398.7 ± 4.8</td>
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<td>Nondiabetic + tranilast</td>
<td>166.0 ± 5.2</td>
<td>170.8 ± 13.9</td>
<td>197.8 ± 19.5*</td>
<td>239.3 ± 21.1*</td>
<td>258.8 ± 20.7*</td>
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<td>Diabetic</td>
<td>162.7 ± 6.8</td>
<td>185.5 ± 12.7*</td>
<td>196.2 ± 24.9*</td>
<td>246.0 ± 25.8*</td>
<td>310.2 ± 20.3*</td>
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<tr>
<td>Diabetic + tranilast</td>
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<td>SBP (mm Hg)</td>
<td>182 ± 19.9</td>
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<td>206.3 ± 16.0</td>
<td>199 ± 8.8</td>
<td>178 ± 10.2</td>
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<tr>
<td>Nondiabetic</td>
<td>168.7 ± 6.1</td>
<td>184.8 ± 7.3</td>
<td>184.2 ± 7.5</td>
<td>178.3 ± 7.1</td>
<td>185.2 ± 7.7</td>
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<td>Nondiabetic + tranilast</td>
<td>167 ± 2.0</td>
<td>188 ± 0.6</td>
<td>190 ± 2.6</td>
<td>186 ± 3.8</td>
<td>169 ± 8.7</td>
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<tr>
<td>Diabetic</td>
<td>147 ± 20.2</td>
<td>200 ± 26.2</td>
<td>183 ± 4.5</td>
<td>187 ± 16.5</td>
<td>187 ± 12.1</td>
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<td>Diabetic + tranilast</td>
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</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>7.3 ± 0.6</td>
<td>6.7 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>6.3 ± 0.3</td>
<td>7.3 ± 0.6</td>
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<tr>
<td>Nondiabetic</td>
<td>6.8 ± 0.2</td>
<td>6.5 ± 0.1</td>
<td>6.2 ± 0.4</td>
<td>6.0 ± 0.1</td>
<td>8.5 ± 1.3</td>
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<td>Nondiabetic + tranilast</td>
<td>7.1 ± 0.9</td>
<td>30.5 ± 1.7*</td>
<td>32.8 ± 0.5*</td>
<td>27.8 ± 3.3*</td>
<td>16.0 ± 3.9*</td>
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<tr>
<td>Diabetic</td>
<td>6.6 ± 0.7</td>
<td>29.8 ± 2.3*</td>
<td>29.0 ± 0.6*</td>
<td>28.3 ± 2.2*</td>
<td>14.9 ± 0.5*</td>
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<td>Diabetic + tranilast</td>
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* p < 0.05 versus nondiabetic.
increased significantly at 24 weeks compared with the values in nondiabetic SHR (nondiabetic and nondiabetic + tranilast). The mesangial area per tuft area (Am/At) was also increased in untreated diabetic SHR. Tranilast treatment ameliorated the increase in mesangial area.

**Type I Collagen mRNA Expression in the Renal Cortex.** To clarify the mechanism by which tranilast inhibited mesangial expansion in diabetic SHR, the renal cortical expression of type I collagen mRNA was estimated by quantitative real-time PCR. Type I collagen mRNA expression was increased by 1.6-fold in untreated diabetic SHR (diabetic) at 24 weeks (p < 0.05; Fig. 3). Tranilast treatment suppressed the increase in type I collagen mRNA expression by 36% in diabetic SHR (p < 0.05 versus diabetic).

**Urinary TGF-β Excretion.** To better understand the mechanism by which tranilast inhibited the accumulation of extracellular matrix in diabetic SHR, urinary excretion of TGF-β, which stimulates collagen synthesis and has a pivotal role in fibrogenesis (Bollineni and Reddi, 1993; Border and Noble, 1994; Sanderson et al., 1995; Sharma and Ziyadeh, 1995), was estimated. Urinary TGF-β excretion increased significantly in untreated diabetic SHR than in nondiabetic SHR from 4 to 24 weeks after the onset of diabetes. At 24 weeks, urinary TGF-β excretion was significantly lower in rats given tranilast compared with untreated diabetic SHR (Fig. 4).

**TGF-β mRNA Expression in the Renal Cortex.** The renal cortical expression of TGF-β mRNA was estimated by quantitative real-time PCR. TGF-β mRNA expression was increased by 2.6-fold in the renal cortex of untreated diabetic SHR (diabetic) compared with nondiabetic SHR (nondiabetic and nondiabetic + tranilast) at 24 weeks (p < 0.05; Fig. 5). Tranilast treatment inhibited the increase of TGF-β mRNA expression by 65% in diabetic SHR (p < 0.05 versus diabetic).

**Immunostaining of TGF-β in the Glomeruli and Tubulointerstitium.** TGF-β protein expression was determined immunohistochemically in the renal glomeruli and tubulointerstitiums. TGF-β immunostaining was much stronger in the untreated diabetic SHR group compared with nondiabetic SHR at 24 weeks (Fig. 6, A–C). Tranilast treatment inhibited the increase in TGF-β immunostaining in diabetic SHR (Fig. 6D).

**Anionic Sites on the LRE.** Representative electron microanionic sites on the LRE of the GBM are shown in Fig. 7. Nondiabetic SHR (nondiabetic and nondiabetic + tranilast) showed a regular distribution of anionic sites (Fig. 7, A and B). In untreated diabetic SHR (diabetic), anionic sites were sparse and irregular compared with those in nondiabetic SHR (Fig. 7C). On the other hand, anionic sites were abundant and regularly arranged in diabetic SHR treated with tranilast (diabetic + tranilast), findings similar to those in nondiabetic SHR group (Fig. 7D). To evaluate the changes in anionic sites quantitatively, we counted the number of sites.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Tuft Area (At) μm²</th>
<th>Glomerular Volume 10⁻⁶ μm²</th>
<th>Mesangial Area (Am) μm²</th>
<th>Am/At</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic</td>
<td>11,491 ± 1905</td>
<td>1.572 ± 0.139</td>
<td>2229 ± 567</td>
<td>0.194 ± 0.033</td>
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<tr>
<td>Nondiabetic + tranilast</td>
<td>11,560 ± 391</td>
<td>1.655 ± 0.117</td>
<td>2128 ± 144</td>
<td>0.187 ± 0.018</td>
</tr>
<tr>
<td>Diabetic</td>
<td>13,840 ± 1285**</td>
<td>2.084 ± 0.151*</td>
<td>3478 ± 210*</td>
<td>0.252 ± 0.020*</td>
</tr>
<tr>
<td>Diabetic + tranilast</td>
<td>10,411 ± 783**</td>
<td>1.335 ± 0.067**</td>
<td>2070 ± 159**</td>
<td>0.200 ± 0.021**</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. nondiabetic; **p < 0.01 vs. diabetic.
per 1000 nm of LRE (Fig. 8). In untreated diabetic SHR, the number of anionic sites was decreased compared with that in nondiabetic SHR ($p < 0.01$). Tranilast treatment completely inhibited the decrease in number of anionic sites and induced an increase to 90% in diabetic SHR ($p < 0.01$ versus untreated diabetic SHR).

**Urinary 8-OHdG Excretion.** Oxidative stress has been reported to cause loss of the charge barrier in isolated kidneys (Kashihaera et al., 1992). To clarify the mechanism by which tranilast inhibited the decrease in number of anionic sites in diabetic SHR, urinary 8-OHdG was measured as a marker of oxidative stress (Shigenaga et al., 1989). Urinary 8-OHdG excretion was significantly increased in untreated diabetic SHR (diabetic) early in the course of diabetes and during the course of experiment, compared with nondiabetic...
Fig. 8. Number of anionic sites per 1000 nm of the lamina rara externa in nondiabetic control SHR (nondiabetic), nondiabetic SHR treated with tranilast (nondiabetic + trani), and diabetic SHR without (diabetic) or with tranilast treatment (diabetic + trani) at 24 weeks. Values represent the means ± S.E.M. of 10 experiments. **, p < 0.01 versus nondiabetic control SHR; ††, p < 0.01 versus untreated diabetic SHR, n = 3 per nondiabetic; n = 3 per nondiabetic + trani; n = 4 per diabetic; and n = 4 per diabetic + trani.

Fig. 9. Time course of changes in urinary 8-OHdG excretion in nondiabetic control rats (nondiabetic), nondiabetic SHR treated with tranilast (nondiabetic + trani), untreated diabetic SHR (diabetic), and diabetic SHR treated with tranilast (diabetic + trani). Values represent the means ± S.E.M. (*, p < 0.05 versus nondiabetic control SHR; †, p < 0.05 versus untreated diabetic SHR).

Discussion

Good glycemic control and blood pressure control are required to prevent the development of diabetic nephropathy (UK Prospective Diabetes Study Group, 1998). Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are known to ameliorate albuminuria in diabetic patients (Parving et al., 2001). Regardless of these interventions, however, diabetic nephropathy continues to progress in a significant proportion of patients and therefore new or additional therapeutic modalities are required. Although the pathogenesis of diabetic nephropathy is multifactorial, it consists mainly of three pathways: 1) glomerular hyperfiltration, 2) expansion of the mesangial matrix, and 3) loss of the charge barrier on the GBM. Of these, the accumulation of extracellular matrix (ECM) is a key determinant. ECM contains different collagen types involved in mesangial expansion (Nerlich and Schleicher, 1991). Recent studies have indicated that hyperglycemia in diabetic nephropathy increases synthesis of ECM components, such as type I and type IV collagen (Glick et al., 1992; Esposito et al., 1996). TGF-β, a potent fibrogenic factor, stimulates collagen synthesis in cultured stellate cells, and hepatic overexpression of TGF-β1 in transgenic mice has been shown to cause hepatic fibrosis (Sanderson et al., 1995; Ikeda et al., 1996). Many studies have demonstrated the increased expansion of TGF-β in the kidneys of diabetic animals and patients, which leads to mesangial matrix expansion and GBM thickening (Bollinieni and Reddi, 1993; Sharma and Ziyadeh, 1995).

In the present study, we examined the role of TGF-β in an experimental model of diabetic nephropathy in SHR through administration of tranilast, a possible inhibitor of TGF-β (Yamada et al., 1994; Miyazawa et al., 1995; Ikeda et al., 1996; Bonnet et al., 2003). Tranilast inhibited TGF-β expression and TGF-β-stimulated matrix synthesis in the glomeruli. Our observations support those of previous reports indicating that tranilast reduced both interstitial fibrosis and tubular atrophy in the kidneys in the unilateral ureteric obstruction model (Miyajima et al., 2001) and that tranilast administration attenuated renal enlargement, albuminuria, and tubulointerstitial fibrosis in an experimental rat model of advanced diabetic nephropathy, and reduced TGF-β-induced hydroxyproline incorporation and fibronectin synthesis in vitro (Mifsud et al., 2003). Mifsud et al. (2003) focused on the effects of tranilast on tubulointerstitial pathology, particularly tubulointerstitial fibrosis and tubular atrophy, not on the glomeruli. In their study, tranilast attenuated tubulointerstitial fibrosis, collagen deposition, and tubular atrophy in vivo. They also found tranilast reduced TGF-β-induced hydroxyproline incorporation and fibronectin synthesis only in vitro. In the present study, we present in vivo evidence of inhibitory effect of tranilast on diabetes-induced overexpression of TGF-β mRNA and protein in the glomeruli. Therefore, tranilast can ameliorate pathological changes not only in the tubulointerstitium but also in the glomeruli. Interestingly, we also found that tranilast ameliorated the progression of diabetic nephropathy by inhibiting glomerular hyperfiltration and loss of the charge barrier on the GBM, despite persistent hyperglycemia and hypertension. Hyperfiltration is recognized as one of the functional changes in the early stages of diabetic nephropathy. In the present study, not only Cr but also tuft area increased in diabetic SHR. Tranilast treatment inhibited both the increase in Cr and expansion of the tuft area in diabetic SHR, suggesting that this agent prevented the progression of hyperfiltration without affecting plasma glucose concentration. It has been reported that increased expression of endothelial cell nitric oxide synthase in afferent arterioles and in glomeruli could cause preferential dilatation of afferent arterioles and ultimately induce glomerular enlargement and hyperfiltration in a diabetic rat model (Sugimoto et al., 1998) and that protein kinase C (PKC)-β inhibitor ameliorated glomerular hyperfiltration in diabetic rats (Ishii et al., 1996). Therefore,
of endothelial cell nitric-oxide synthase and PKC may be targets of tranilast involved in amelioration of glomerular hyperfiltration.

This is the first report to describe an effect of tranilast on anionic sites. We assumed that the preventive effect of tranilast on the loss of anionic sites is one of the major factors involved in the amelioration of albuminuria in diabetic rats (Yamashita et al., 2002; Ota et al., 2003). Anionic sites consist of glycosaminoglycans and are rich in heparan sulfate proteoglycans (HSPG) (Kanwar and Farquhar, 1979). These anionic sites of the GBM regulate the transudation of circulating macromolecules across the GBM as a charge-selective filtration barrier; therefore, their loss leads to an increase in the permeation of anionic proteins, such as ferritin and albumin (Kanwar et al., 1980; Rosenzweig and Kanwar, 1982).

It has been reported that hyperglycemia decreases both the production and sulfation of HSPG by reducing the activity of glucosaminyl N-deacetylase, a key enzyme in HSPG synthesis (Kofod-Enevoldsen, 1992). Moreover, oxidative stress has been suggested to cause loss of the charge barrier in isolated kidneys (Kashihara et al., 1992). It has been reported that diabetes causes oxidative DNA damage (Danford et al., 1996) and accumulation of 8-OHdG in diabetic patients causes more advanced vascular complications (Hinojko et al., 1999). In the present study, urinary 8-OHdG excretion was significantly decreased in rats given tranilast compared with untreated diabetic SHR. Thus, tranilast could inhibit the loss of anionic sites by reducing oxidative stress.

Recently, it was shown that normalizing levels of mitochondrial reactive oxygen species prevents the three major pathways caused by hyperglycemia: PKC, advanced glycation end products (AGEs), and the aldose reductase pathway (Nishikawa et al., 2000). PKC and AGEs have been shown to increase TGF-β expression in mesangial cells (Yang et al., 1994; Studer et al., 1995). Oxidative stress may stimulate TGF-β-stimulated matrix synthesis in the glomeruli through the activation of PKC and AGE-mediated pathways. Moreover, oxidative stress could directly cause loss of the charge barrier on the GBM. Indeed, urinary 8-OHdG excretion was significantly decreased in rats given tranilast compared with untreated diabetic SHR. Thus, tranilast could inhibit the loss of anionic sites by reducing oxidative stress.

In conclusion, the results of the present study indicated that an antihypertensive drug, tranilast, prevented the development of nephropathy in diabetic SHR by 1) inhibiting diabetes-induced up-regulation of TGF-β expression and mesangial expansion; 2) ameliorating glomerular hyperfiltration; and 3) by inhibiting loss of the charge barrier on GBM, without affecting plasma glucose concentration or blood pressure. Our results suggested that tranilast may be clinically useful in the treatment of diabetic nephropathy due to its pleiotropic effects.

References


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