17β-Estradiol attenuates diabetic kidney disease by regulating extracellular matrix and transforming growth factor-β protein expression and signaling

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Submitted 14 February 2007; accepted in final form 2 August 2007

Dixon A, Maric C. 17β-Estradiol attenuates diabetic kidney disease by regulating extracellular matrix and transforming growth factor-β protein expression and signaling. Am J Physiol Renal Physiol 293: F1678–F1690, 2007. First published August 8, 2007; doi:10.1152/ajprenal.00079.2007.—We previously showed that supplementation with 17β-estradiol (E2) from the onset of diabetes attenuates the development of diabetic renal disease. The aim of the present study was to examine whether E2 can also attenuate the disease process once it has developed. The present study was performed in nondiabetic and streptozotocin-induced diabetic Sprague-Dawley rats. E2 supplementation began after 9 wk of diabetes and continued for 8 wk. Diabetes was associated with an increase in urine albumin excretion, glomerulosclerosis, tubulointerstitial fibrosis, renal cortical collagen type I and IV, laminin, plasminogen activator inhibitor-1, tissue inhibitors of metalloproteinase-1 and -2, transforming growth factor-β, tissue inhibitors of metalloproteinase-1 and -2, transforming growth factor-β (TGF-β) type I and II, Smad2/3, phosphorylated Smad2/3, and Smad4 protein expression, and CD68-positive cell abundance. Decreases in matrix metalloproteinase (MMP)-2 protein expression and activity and decreases in Smad6 and Smad7 protein expression were also associated with diabetes. E2 supplementation completely or partially attenuated all these changes, except Smad4 and fibronectin, on which E2 supplementation had no effect. These data suggest that E2 attenuates the progression of diabetic renal disease once it has developed by regulating extracellular matrix, TGF-β, and expression of its downstream regulatory proteins. These findings support the notion that sex hormones in general, and E2 in particular, are important regulators of renal function and may be novel targets for the treatment and prevention of diabetic renal disease.

diabetes; estradiol; fibrosis; glomerulosclerosis; transforming growth factor-β; Smads

Although the female sex appears to be a protective factor against the development of nondiabetic renal disease (32, 36, 46), this protection is not as apparent in the setting of diabetes. The incidence and the rate of progression of renal disease are far greater in diabetic than in nondiabetic women (6, 36), suggesting that the diabetic milieu may provide a stage for the development and faster progression of renal disease in women. Our previous studies suggested that reduced levels of plasma estradiol and abnormal regulation of estrogen receptors in the diabetic kidney might account for the loss of the female sex as a protective factor in diabetes (27, 51). Furthermore, these studies showed that supplementation with 17β-estradiol (E2) from the onset of diabetes restored the levels of estradiol to those observed in nondiabetic rats and protected against the development of diabetic renal injury (27). One of the mechanisms through which E2 exerts this renoprotection is by regulating extracellular matrix (ECM) protein expression (26, 27).

Increased ECM protein synthesis and/or decreased ECM degradation contributes to the development of diabetes-associated glomerulosclerosis and tubulointerstitial fibrosis (29, 53). It is well recognized that the rate of progression of diabetic renal disease correlates with the degree of cortical tubulointerstitial fibrosis (15, 34). Thus, attenuating ECM accumulation and/or enhancing ECM degradation is considered a prime target in the treatment of diabetic renal complications.

Transforming growth factor-β (TGF-β) is a key regulator of ECM protein synthesis and degradation in the diabetic kidney (8, 50). TGF-β has consistently been shown to be upregulated in renal parenchymal and infiltrating inflammatory cells in the diabetic kidney in humans and experimental models (3, 55). A number of factors, including hyperglycemia (44), advanced glycation end products (52), oxidative stress (47), and angiotensin (9), have been shown to stimulate TGF-β protein expression in the diabetic kidney. TGF-β exerts its effects, first, through binding to the membrane-bound TGF-β type II receptor (TGF-βRII) and, then, through activation of the TGF-β type I receptor (TGF-βRI) (5, 50). This receptor activation is followed by activation of the Smad regulatory pathway: Smad2 and Smad3 are phosphorylated and form a heteromeric complex with Smad4, and then the complex translocates into the nucleus, where it regulates transcription of target genes (33). In contrast, Smad6 and Smad7 are inhibitory Smads that act in a negative-feedback loop to inhibit TGF-β activity by preventing phosphorylation of Smad proteins and activation of TGF-βRI (50).

Our previous studies showed that supplementation with E2 from the onset of diabetes prevents albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis associated with diabetic nephropathy via regulation of ECM synthesis and degradation (26, 27). Although these studies show that E2 is able to attenuate diabetic renal injury when supplemented from the onset of diabetes, little is known about the ability of E2 to attenuate the progression of the disease once it has developed. This is of particular importance, since the vast majority of patients with diabetes present with moderate or advanced renal injury; thus attenuating the disease progression is of high clinical relevance. The aim of the present study was to examine whether E2 is also able to attenuate the progression of renal functional and structural changes once they have been initiated by the diabetic milieu. Specifically, our study examined the

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effects of E2 supplementation on ECM protein expression and the expression of TGF-β and its downstream signaling proteins involving Smads in the streptozotocin (STZ)-induced model of diabetic nephropathy.

MATERIALS AND METHODS

Animal model. Female Sprague-Dawley rats (10 wk of age; Harlan, Madison, WI) were maintained on a phytoestrogen-free rat chow (Harlan, Madison, WI) and tap water ad libitum. The animals were first randomly divided into two treatment groups: nondiabetic (ND, n = 8) and diabetic (D, n = 14) for 9 wk. The diabetic animals were then further randomly divided into two treatment groups: diabetic (D, n = 7) and diabetic with E2 supplementation (D + E2, n = 7). All animals, including the ND group, were treated for an additional 8 wk. After a total of 17 wk, the animals were weighed and anesthetized with sodium pentobarbitone (40 mg/kg ip), and blood was collected (via cardiac puncture) for measurement of plasma E2 levels. The

Table 1. Dilutions, sources, and manufacturers of antibodies used for immunohistochemistry and Western blot

<table>
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<tr>
<th>Antibody</th>
<th>Ab Dilution for IHC</th>
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<th>Ab Dilution for Western Blot</th>
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<td>Smad2/3 (30 μg)</td>
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<td>Smad6 (30 μg)</td>
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IHC, immunohistochemistry; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; PAI, plasminogen activator inhibitor; TGF, transforming growth factor; pSmad2/3, phosphorylated Smad2/3; N/A, not applicable.

Fig. 1. A: glomerulosclerosis in periodic acid-Schiff-stained sections of renal cortex. B: tubulointerstitial fibrosis in Masson’s trichrome-stained sections of renal cortex. E2, 17β-estradiol; g, glomerulus; arrowhead, mesangial expansion. Original magnification ×400.
Induction of diabetes. After an overnight fast, the animals received a single intraperitoneal injection of 0.1 M citrate buffer (pH 4.5, ND group) or 55 mg/kg STZ (Sigma, St. Louis, MO) in 0.1 M citrate buffer (D and D + E2 groups). Diabetic rats were supplemented with insulin (2–4 U sc every 2nd day; Lantus, Aventis Pharmaceuticals, Kansas City, MO) for the duration of the study to maintain blood glucose levels at 250–450 mg/dl and prevent weight loss. Every 4 wk, the animals were placed in metabolic cages for 24 h for determination of urine output and measurement of urinary albumin excretion (UAE).

E2 supplementation and plasma estradiol levels. At the time of induction of diabetes, the animals were subjected to two intraperitoneal injections (4 h apart) of deslorelin acetate (10 mg/ml; Bachem Chemicals, Torrance, CA). The D + E2 group was injected with E2 (5 mg/kg in 200 l of peanut oil; Sigma) every 4 days to mimic the cyclical nature of estrogen release, whereas non-E2-supplemented animals were injected with 200 l of peanut oil only. The dose of E2 was chosen on the basis of our previous studies showing that this dose, when injected every 4 days, results in circulating E2 levels that are in the peak physiological range (51). Plasma E2 levels were measured by ELISA (Alpha Diagnostics, San Antonio, TX) according to the manufacturer’s protocol.

UAE. Urine albumin concentration was determined using the Nephrat II albumin kit (Exocell, Philadelphia, PA) according to the manufacturer’s protocol. The rate of UAE was calculated from the measurement of urine albumin concentration and output.

Table 2. Effects of E2 supplementation on metabolic and renal parameters

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>D</th>
<th>D + E2</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>258.4±14</td>
<td>250±3.5</td>
<td>223±13</td>
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<tr>
<td>Kidney wt, g</td>
<td>1.4±0.10</td>
<td>1.5±0.17</td>
<td>1.4±0.10</td>
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<td>Blood glucose, mg/dl</td>
<td>109.3±8.9</td>
<td>498.3±30.1‡</td>
<td>446.6±23.0£‡</td>
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<tr>
<td>Plasma E2, pg/ml</td>
<td>54.9±4.6</td>
<td>40.2±4.1*</td>
<td>53.1±6.4§</td>
</tr>
<tr>
<td>UAE, mg/day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 wk</td>
<td>6.6±0.4</td>
<td>19.4±3.1†</td>
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<tr>
<td>17 wk</td>
<td>9.4±1.0</td>
<td>56.2±8.9‡</td>
<td>39.4±15.7†‡</td>
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<tr>
<td>GSI, AU</td>
<td>0.13±0.02</td>
<td>1.38±0.09†</td>
<td>0.20±0.02§</td>
</tr>
<tr>
<td>TIFI, AU</td>
<td>0.17±0.04</td>
<td>1.35±0.10†</td>
<td>0.38±0.07†</td>
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</tbody>
</table>

Values are means ± SE. E2, 17β-estradiol; ND, nondiabetic; D, diabetic, D + E2, E2-supplemented diabetic; UAE, urine albumin excretion; GSI, glomerulosclerosis index; TIFI, tubulointerstitial fibrosis index; AU, arbitrary units. *P < 0.05; †P < 0.01; ‡P < 0.001 vs. ND. §P < 0.05 vs. D.

Fig. 2. Collagen type I and type IV renal cortical immunolocalization and protein expression. Top: collagen type I and type IV protein expression in representative sections of renal cortex. pt, Proximal tubule; dt, distal tubule; arrowhead, mesangial cell. Original magnification ×400. Bottom: densitometric scans of collagen type I and type IV protein levels expressed as ratio of collagen type I to Coomassie blue and ratio of collagen type IV to Coomassie blue in relative optical density (ROD) units. Values are means ± SE. ND, nondiabetic; D, diabetic; D + E2, 17β-estradiol (E2)-supplemented diabetic.
Indexes of glomerulosclerosis and tubulointerstitial fibrosis. Glomerulosclerosis is defined as accumulation of ECM deposits and mesangial expansion (2). The glomerulosclerosis index (GSI) was assessed in periodic acid-Schiff-stained sections in 80 randomly selected glomeruli, and the degree of sclerosis was graded using a semiquantitative scoring method, as previously described (42). Tubulointerstitial fibrosis is defined as tubular atrophy or dilatation, deposition of ECM, and presence of inflammatory cells (2). The tubulointerstitial fibrosis index (TIFI) was assessed in Masson’s trichrome-stained sections in 60 randomly selected fields of view at ×400 magnification using a light microscope, and the degree of fibrosis was graded using a semiquantitative scoring method, as previously described (27).

Immunohistochemistry. In 4-μm-thick paraffin sections, endogenous avidin and biotin activities were blocked using a kit (Vector, Burlingame, CA). Sections were incubated with 0.1% albumin (for collagen types I and IV) or 10% nonimmune goat serum (for other proteins) in PBS (pH 7.4) for blocking of nonspecific immunolabeling. Sections were then incubated with antisera against proteins of interest (specific concentrations are shown in Table 1) at 4°C overnight. After the sections were washed with PBS, they were incubated with biotinylated goat anti-rabbit, anti-mouse, or anti-goat IgG (Dakopatts, Glostrup, Denmark) diluted 1:100 in PBS for 1 h at room temperature and then with the avidin-biotin complex (Vector) diluted 1:100 with PBS for 1 h at room temperature. Positive immunoreaction was detected after incubation with 3,3-diaminobenzidine for 2 min at room temperature and counterstaining with Mayer’s hematoxylin. Sections incubated with 0.1% albumin or 10% goat serum, instead of the primary antisera, were used as negative controls.

Western blotting. Renal cortical tissue samples were homogenized, and the protein concentration was determined using a colorimetric assay (Bio-Rad). For collagen types I and IV, samples were analyzed under nonreducing conditions; for other proteins, samples were denatured at 95°C for 10 min and then treated as follows. After incubation with primary antisera at 4°C overnight, the membranes were washed and incubated with goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase, and proteins were visualized by enhanced chemiluminescence (KPL, Gaithersburg, MD). The densities of specific bands were normalized to the total amount of protein loaded in each well after densitometric analysis of gels stained with Coomassie blue. The densities of specific bands were quantitated by densitometry using Scion Image Beta software.

Zymography. Renal cortical tissue samples were homogenized, and the protein concentration was determined using a colorimetric assay (Bio-Rad). The homogenized samples were loaded onto a 10% SDS-acrylamide gel containing 1 mg/ml gelatin (Bio-Rad). After electrophoresis, the gel was incubated in renaturing buffer (Invitrogen, Carlsbad, CA) for 30 min at 37°C. After the gel had been dyed with Coomassie blue, it was air dried. After hydration, the gel was incubated in a renaturing buffer containing 50 μg/ml of TGF-β (R&D Systems, Minneapolis, MN) for 24 h at 37°C. Following incubation, the gel was washed in a solution of TGF-β extraction buffer, and bands for pro- and mature collagenases were visualized by enhanced chemiluminescence (KPL). The densities of specific bands were quantitated by densitometry using Scion Image Beta software.

Fig. 3. Laminin and fibronectin renal cortical immunolocalization and protein expression. Top: laminin and fibronectin protein expression in representative sections of renal cortex. Arrow, tubulointerstitial areas; see Fig. 2 legend for other abbreviations. Original magnification ×400. Bottom: densitometric scans of laminin and fibronectin protein levels expressed as ratio of laminin to Coomassie blue and ratio of fibronectin to Coomassie blue. Values are means ± SE.
Carlsbad, CA) and activated in developing buffer (Invitrogen), and gelatinase activity was visualized by staining with Coomassie blue. Bands were quantitated by densitometry using Scion Image Beta (version 4.02) software.

Quantitative analysis of macrophage number. The number of macrophages (CD68-positive cells) was assessed by counting the number of positive cells in 20 random fields per section in 4 sections per kidney from each treatment group.

Statistical analysis. Values are means ± SE. Data were analyzed with a one-way ANOVA followed by Tukey’s post test using Sigma Stat software. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Body and kidney weight, blood glucose and $E_2$ levels, and UAE. No differences in body weight or kidney weight were observed between any of the treatment groups (Table 2). Diabetes was associated with increased blood glucose levels ($109.3 ± 8.9$ and $498.3 ± 30.1$ mg/dl in ND and D groups, respectively, $P < 0.001$), decreased plasma levels of $E_2$ ($54.9 ± 4.6$ and $40.2 ± 4.1$ pg/ml in ND and D groups, respectively, $P < 0.05$), and increased UAE ($9.4 ± 1.0$ and $56.2 ± 8.9$ mg/day in ND and D groups, respectively, $P < 0.001$). The D + $E_2$ group exhibited an increase in blood glucose levels similar to that in the D group ($446.6 ± 23.0$ mg/dl), whereas the decrease in plasma levels of $E_2$ ($53.1 ± 6.4$ pg/ml) and increase in UAE ($39.4 ± 15.7$ mg/day) associated with diabetes were completely (plasma $E_2$) or partially (UAE) attenuated in the D + $E_2$ group (Table 2).

UAE was also measured after the initial 9 wk of diabetes, i.e., before $E_2$ supplementation. At 9 wk, UAE was increased in the D group ($6.6 ± 0.4$ and $19.4 ± 3.1$ mg/day in ND and D groups, respectively, $P < 0.01$; Table 2).

Glomerulosclerosis and tubulointerstitial fibrosis. Diabetes was associated with moderate glomerulosclerosis [GSI = $0.13 ± 0.02$ and $1.38 ± 0.09$ arbitrary units (AU) in ND and D groups, respectively, $P < 0.01$; Fig. 1A, Table 2] and...

Fig. 4. Matrix metalloproteinase (MMP)-2 and MMP-9 renal cortical immunolocalization, protein expression, and activity. Top: MMP-2 and MMP-9 protein expression in representative sections of renal cortex. Original magnification ×400. Bottom: densitometric scans of MMP-2 and MMP-9 protein levels expressed as ratio of MMP-2 to Coomassie blue and ratio of MMP-9 to Coomassie blue. Values are means ± SE.
tubulointerstitial fibrosis (TIFI = 0.17 ± 0.04 and 1.35 ± 0.09 AU in ND and D groups, respectively, $P < 0.01$; Fig. 1B, Table 2). In the D + E$_2$ group, these changes were attenuated (GSI = 0.20 ± 0.02 AU and TIFI = 0.38 ± 0.078 AU; Fig. 1, Table 2).

Collagen types I and IV, laminin, and fibronectin. In the ND kidney, collagen type I was mainly immunolocalized to tubulointerstitial areas of the renal cortex (Fig. 2), whereas collagen type IV (Fig. 2) and laminin and fibronectin (Fig. 3) were predominantly immunolocalized to basement membranes of the glomerulus and proximal and distal tubules. Diabetes was associated with an overall increase in the intensity of immunostaining for collagen types I and IV (Fig. 3) and laminin and fibronectin (Fig. 4), whereas E$_2$ supplementation completely or partially attenuated these changes. Quantitation of changes in collagen types I and IV and laminin and fibronectin protein expression by Western blotting confirmed the immunohistochemical observations. Diabetes was associated with an increase in collagen type I [1.02 ± 0.05 and 1.83 ± 0.03 relative optical density units (ROD) in ND and D groups, respectively, $P < 0.001$], collagen type IV (0.97 ± 0.05 and 1.56 ± 0.03 ROD in ND and D groups, respectively, $P < 0.01$), and laminin (1.03 ± 0.03 and 1.80 ± 0.02 ROD in ND and D groups, respectively, $P < 0.001$) protein expression (Figs. 3 and Fig. 4) but no changes in fibronectin protein expression (Fig. 4). In the D + E$_2$ group, changes in collagen type I and laminin were partially attenuated (1.26 ± 0.10 and 1.45 ± 0.01 ROD, respectively) and changes in collagen type IV were fully attenuated (0.86 ± 0.32 ROD).

Matrix metalloproteinases, tissue inhibitors of metalloproteinases, and plasminogen activator inhibitor-1. No apparent differences in the intensity of immunostaining for matrix metalloproteinase (MMP)-2 or MMP-9 were observed between the D and ND groups (Fig. 5); however, the intensity of immunostaining for MMP-2 in the D + E$_2$ group appeared to increase above that in the ND group. Quantitative analysis of MMP-2 protein expression and activity by Western blotting and zymography, respectively, showed a decrease in MMP-2 protein expression (1.35 ± 0.06 and 0.98 ± 0.05 ROD in ND and D groups, respectively, $P < 0.05$) and activity (0.39 ± 0.09 and 0.22 ± 0.01 ROD in ND and D groups, respectively, $P < 0.05$) associated with diabetes but no differences in

Fig. 5. Tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2, and plasminogen activator inhibitor-1 (PAI-1) renal cortical immunolocalization and protein expression. Top: TIMP-1, TIMP-2, and PAI-1 protein expression in representative sections of renal cortex. Original magnification ×400. **Bottom:** densitometric scans of TIMP-1, TIMP-2, and PAI-1 protein levels expressed as ratio of TIMP-1 to Coomassie blue, ratio of TIMP-2 to Coomassie blue, and ratio of PAI-1 to Coomassie blue. Values are means ± SE.
MMP-9 protein expression or activity (Fig. 5). MMP-2 protein expression and activity in the D\textsuperscript{+}E\textsubscript{2} group were increased to levels above or similar to those in the ND group (Fig. 5).

Although tissue inhibitor of metalloproteinase (TIMP)-1 was not detectable by immunohistochemistry, TIMP-2 was mainly immunolocalized to distal tubules (Fig. 5). Quantitative analysis of TIMP-1 and TIMP-2 protein expression by Western blotting (Fig. 5) showed that diabetes was associated with an increase in TIMP-1 (0.30 \pm 0.07 and 0.93 \pm 0.14 ROD in ND and D groups, respectively, \( P < 0.01 \)) and TIMP-2 (0.40 \pm 0.09 and 0.96 \pm 0.11 ROD in ND and D groups, respectively, \( P < 0.01 \)) protein expression. These changes were completely attenuated in the D\textsuperscript{+}E\textsubscript{2} group.

Similar to TIMPs, the intensity of plasminogen activator inhibitor-1 (PAI-1) immunostaining in proximal and distal tubules (Fig. 5) and protein expression (0.34 \pm 0.08 and 0.88 \pm 0.03 ROD in ND and D groups, respectively, \( P < 0.01 \)) were increased with diabetes. These changes were partially attenuated in the D\textsuperscript{+}E\textsubscript{2} group.

**TGF-\( \beta \), TGF-\( \beta \)RI, and TGF-\( \beta \)RII protein expression.** Although only weak immunostaining for TGF-\( \beta \) was observed in the ND group, the intensity of immunostaining in glomerular mesangial cells and proximal and distal tubules was increased in the D group (Fig. 6). This diabetes-associated increase in the intensity of TGF-\( \beta \) immunostaining was attenuated in the D\textsuperscript{+}E\textsubscript{2} group. Western analysis confirmed the immunohistochemical findings. Diabetes was associated with an increase in renal cortical expression of TGF-\( \beta \) protein (0.70 \pm 0.14 and 1.63 \pm 0.08 ROD in ND and D groups, respectively, \( P < 0.001 \)); this increase was partially attenuated in the D\textsuperscript{+}E\textsubscript{2} group (1.27 \pm 0.10 ROD; Fig. 7).

In the ND group, TGF-\( \beta \)RI was predominantly immunolocalized to glomerular mesangial cells (Fig. 6). An apparent increase in the overall intensity of TGF-\( \beta \)RI immunostaining was observed in the D group in the mesangial cells as well as in distal and, to a lesser extent, proximal tubules (Fig. 6). This diabetes-associated increase in the intensity of TGF-\( \beta \)RI immunostaining was attenuated in the D\textsuperscript{+}E\textsubscript{2} group. Although TGF-\( \beta \)RII was not detectable in the ND group by immunohistochemistry, it was immunolocalized predominantly to proximal and distal tubules and in the mesangial cells in the D group (Fig. 6). This diabetes-associated increase in the intensity of TGF-\( \beta \)RII immunostaining was attenuated in the D\textsuperscript{+}E\textsubscript{2} group. Western analysis confirmed the immunohistochemical findings. Diabetes was associated with an increase in renal cortical expression of TGF-\( \beta \)RII (0.69 \pm 0.23 and 1.47 \pm 0.14 ROD in ND and D groups, respectively, \( P < 0.05 \)) and TGF-\( \beta \)RII (0.40 \pm 0.09 and 1.07 \pm 0.10 ROD in ND and D groups, respectively, \( P < 0.001 \)) protein; this increase was partially

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**Fig. 6.** Transforming growth factor (TGF)-\( \beta \) and TGF-\( \beta \) receptor type I and II (TGF-\( \beta \)RI and TGF-\( \beta \)RII) immunolocalization. Original magnification \( \times 400 \).
attenuated in the D + E2 group (0.97 ± 0.12 and 0.68 ± 0.04 ROD for TGF-βRI and TGF-βRII, respectively; Fig. 7).

**Smad protein expression.** In the ND group, no Smad2/3, phosphorylated Smad2/3 (pSmad2/3), or Smad4 was detected by immunohistochemistry (Fig. 8). In the D group, Smad2/3, pSmad2/3, and Smad4 were immunolocalized to glomerular mesangial cells and proximal and distal tubules (Fig. 8). This diabetes-associated increase in the intensity of Smad2/3, pSmad2/3, and Smad4 immunostaining was attenuated in the D + E2 group. Western analysis confirmed the immunohistochemical findings. Diabetes was associated with an increase in renal cortical expression of Smad2/3 (0.68 ± 0.08 and 1.71 ± 0.03 ROD in ND and D groups, respectively, \( P < 0.01 \); Fig. 9), pSmad2/3 (0.28 ± 0.07 and 0.74 ± 0.12 ROD in ND and D groups, respectively, \( P < 0.05 \); Fig. 9), and Smad4 (0.82 ± 0.24 and 1.77 ± 0.03 ROD in ND and D groups, respectively, \( P < 0.05 \); Fig. 9) protein. These changes were attenuated in the D + E2 group (1.24 ± 0.19, 0.24 ± 0.09, and 1.65 ± 0.03 ROD for Smad2/3, pSmad2/3, and Smad4, respectively; Fig. 9).

In the ND group, Smad6 was mainly immunolocalized to proximal and, to lesser extent, distal tubules (Fig. 10), whereas Smad7 was immunolocalized predominantly to distal tubules (Fig. 10). In the D group, an overall decrease in the intensity of immunolocalization for Smad6 and Smad7 was observed (Fig. 10). This diabetes-associated decrease in the intensity of Smad6 and Smad7 immunostaining was attenuated in the D + E2 group. Western analysis confirmed the immunohistochemical findings. Diabetes was associated with a decrease in renal cortical expression of Smad6 (0.85 ± 0.10 and 0.40 ± 0.03 ROD in ND and D groups, respectively, \( P < 0.001 \)) and Smad7 (1.41 ± 0.06 and 1.01 ± 0.05 ROD in ND and D groups, respectively, \( P < 0.05 \)) protein (Fig. 10). These changes were attenuated in the D + E2 group (0.70 ± 0.07 and 1.27 ± 0.09 ROD for Smad6 and Smad7, respectively).

**DISCUSSION**

We previously showed that E2, when supplemented from the onset of diabetes, is renoprotective by preventing albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis (26, 27). The present study demonstrates that when supplemented after 9 wk of diabetes, E2 is also able to attenuate albuminuria and structural changes associated with diabetes through regulation of ECM protein and TGF-β protein expression and signaling.

Increased UAE is a hallmark of diabetic renal disease (7). In the present study, increased UAE was observed as early as 9 wk of diabetes and increased further for the duration of the study (17 wk). E2 supplementation initiated after 9 wk of diabetes, when albuminuria has already developed, reduced this diabetes-associated increase in UAE. These observations suggest that E2 attenuates, as well as reverses, the diabetes-associated decline in albuminuria. Variable effects of E2 sup-

![Fig. 7. TGF-β, TGF-βRI, and TGF-βRII protein expression. Top: representative gels of TGF-β, TGF-βRI, and TGF-βRII protein. Bottom: densitometric scans of TGF-β, TGF-βRI, and TGF-βRII protein levels expressed as ratio of TGF-β to Coomassie blue, ratio of TGF-βRI to Coomassie blue, and ratio of TGF-βRII to Coomassie blue. Values are means ± SE.](http://ajprenal.physiology.org/)

![Fig. 10. Smad6 and Smad7 protein expression.](http://ajprenal.physiology.org/)
plementation on UAE have been reported in other studies. Although E2 had no effect on the Otsuka Long-Evans Tokushima fatty diabetic rat (49), in type 2 diabetic patients, E2 supplementation from the onset of diabetes reduced proteinuria (48). However, no studies have examined the ability of E2 to reverse diabetes-related albuminuria.

Our previous studies showed that E2 supplementation from the onset of diabetes prevents the development of glomerulosclerosis and tubulointerstitial fibrosis (26, 27). Our previous study also shows that the predominant receptor type through which E2 exerts its effects in the diabetic kidney is estrogen receptor-α (51). The present study shows that when E2 is supplemented after 9 wk of diabetes, it also attenuates the progression of development of glomerulosclerosis and tubulointerstitial fibrosis. Specifically, we show that a 17-wk period of diabetes is associated with an upregulation of collagen types I and IV, laminin, and fibronectin protein expression, confirming observations of previous studies from this and other laboratories (20, 25, 26). Supplementation with E2 partially (collagen type IV and laminin) or fully (collagen type I) attenuated these increases but did not affect fibronectin protein expression. Most studies mainly examined the ability of E2 to regulate renal ECM protein expression (4, 10, 13, 28). It was surprising that E2 was unable to attenuate the diabetes-associated increase in fibronectin protein expression, in that our previous study showed that, when supplemented from the onset of diabetes, E2 attenuated fibronectin protein expression (26). One explanation for this observation may be the inability of E2 to adequately upregulate enzymes involved in fibronectin metabolism. Although E2 upregulates expression of MMP-2, fibronectin degradation, with advanced diabetes, may be more dependent on other MMPs, including MMP-9, which are not responsive to E2.

In addition to increased synthesis, diabetes-related glomerulosclerosis and tubulointerstitial fibrosis are associated with a decrease in ECM degradation by MMPs (24, 29). Our study shows that a 17-wk period of diabetes is associated with an upregulation of collagen types I and IV, laminin, and fibronectin protein expression, confirming observations of previous studies from this and other laboratories (20, 25, 26). Supplementation with E2 partially (collagen type IV and laminin) or fully (collagen type I) attenuated these increases but did not affect fibronectin protein expression. Most studies mainly examined the ability of E2 to regulate renal ECM protein expression in vitro (12, 40, 45). The few in vivo studies, albeit mostly performed in non-diabetic models, support the observation of the present study that E2 downregulates ECM protein expression (4, 10, 13, 28). It was surprising that E2 was unable to attenuate the diabetes-associated increase in fibronectin protein expression, in that our previous study showed that, when supplemented from the onset of diabetes, E2 attenuated fibronectin protein expression (26). One explanation for this observation may be the inability of E2 to adequately upregulate enzymes involved in fibronectin metabolism. Although E2 upregulates expression of MMP-2, fibronectin degradation, with advanced diabetes, may be more dependent on other MMPs, including MMP-9, which are not responsive to E2.

Variable levels of MMP-2 and MMP-9 protein expression and activity have been observed in the diabetic kidney, depending on the duration and model of diabetes. In early diabetes, a decrease in MMP-2 and MMP-9 protein expression and activity have been reported (17, 26, 31); in longer-term diabetes, no changes in MMP-9 activity were observed (11, 41). It is conceivable that compensatory mechanisms are activated in response to pro-

**Fig. 8.** Smad2/3, phosphorylated Smad2/3 (pSmad2/3), and Smad4 immunolocalization. Original magnification ×400.
longed diabetes that reverse MMP-9 expression and activity in early stages of the disease. Moreover, studies in MMP-9-knockout mice showed that MMP-9 does not appear to have a discernible role in the progression of glomerulonephritis (1), suggesting compensation or redundancy of MMP-9 in this model. MMP-9 may similarly be redundant or compensated in the diabetic kidney, especially after prolonged hyperglycemia. Similar to ECM protein expression, very few in vivo studies have examined the ability of E2 to regulate renal ECM metabolism, especially in the setting of diabetes. In cultured mesangial cells, E2 increases MMP-2 (16, 22) and hyperglycemia-induced downregulation of MMP-9 (39) protein expression and activity. These observations support the findings of the present study that E2 is renoprotective by upregulating MMP expression and activity.

One of the mechanisms by which MMP activity is regulated is by altering the expression of its inhibitors, namely, TIMPs and PAI-1. Diabetic nephropathy in humans and experimental models is associated with increased TIMP-1 (31), TIMP-2 (17), and PAI-1 (37) protein expression, consistent with the findings of the present study. Although E2 supplementation partially (TIMP-1) or fully (TIMP-2) attenuated the diabetes-associated increase in TIMPs, no effect of E2 on PAI-1 expression was observed. In previous studies, E2 was shown to attenuate TIMP-2 and PAI-1 expression in the Alb/TGF-β transgenic mice (4) and attenuate TIMP-1 and TIMP-2 in the diabetic kidney (26). Furthermore, combined estrogen and medroxyprogesterone treatment in postmenopausal diabetic women reduces serum PAI-1 levels (38). These studies clearly demonstrate the ability of E2 to regulate PAI-1 expression; however, it appears that this regulation is ineffective once diabetes has progressed, at least in the kidney. Despite the inability of E2 to regulate PAI-1 expression directly, its effects on other ECM regulatory pathways appear to be sufficient to effectively attenuate the progression of glomerulosclerosis and tubulointerstitial fibrosis in the diabetic kidney.

Numerous studies have demonstrated the importance of TGF-β in the pathophysiology of renal disease, including diabetic nephropathy. The diabetic kidney is characterized by temporal overexpression of TGF-β mRNA and protein in experimental models (3) and humans (55). Our previous study (27) and the present study confirm that TGF-β is overexpressed in glomerular mesangial cells and proximal and distal tubules in the STZ-induced diabetic rat after 9 wk of diabetes. Our present study also shows that supplementation with E2 for 8 wk following the 9-wk period of diabetes attenuates TGF-β protein expression, suggesting that one of the mechanisms by which E2 exerts its renoprotective effects in the diabetic kidney is regulation of the expression of locally active cytokines, such as TGF-β. A similar renoprotective effect of E2 has been observed in the five-sixths remnant kidney model, in which E2 supplementation inhibits tubulointerstitial fibrosis via a reduction of TGF-β expression (21).

Observations from the present study indicate that TGF-βRII and TGF-βRI protein expression is upregulated in the diabetic kidney, supporting previously reported findings (18, 23). Our study also shows that E2 supplementation reduces TGF-βRII and TGF-βRI protein expression, suggesting that E2 exerts its renoprotective effects by regulating expression of the ligand (TGF-β) and its receptors. Although in vivo (4, 21) and in vitro (35) studies have shown that E2 downregulates TGF-β in the setting of diabetes/high glucose, no studies have reported the effects of E2 on TGF-βRII and TGF-βRI protein expression.
Our observations thus demonstrate a novel aspect of E2 regulation of the TGF-β pathway in the diabetic kidney.

Evidence suggests that TGF-β exerts its profibrotic effect through activation of Smad2/3, which forms a complex with Smad4 that is translocated into the nucleus to upregulate transcription of profibrotic genes (33, 50). According to the present study, diabetes is associated with an increase in the renal cortical expression of Smad2/3 protein as well as its phosphorylated form, pSmad2/3, indicating increased activation of Smad2/3 in the setting of diabetes. Other studies have also reported increases in renal cortical Smad2/3 and Smad4 in the diabetic kidney (14, 19). The present study shows that E2 supplementation attenuates diabetes-associated increases in Smad2/3 and pSmad2/3 protein expression but has no effects on Smad4. In cultured human embryonic kidney carcinoma cells, E2 also decreases Smad3 protein expression (30), further supporting the notion that E2, in addition to being able to directly regulate TGF-β protein expression, also suppresses TGF-β activity by downregulating its profibrotic regulatory proteins Smad2/3. Although E2 did not affect Smad4 protein expression, it is conceivable that E2 suppresses the profibrotic TGF-β effect by downregulating Smad2/3 protein expression and its phosphorylation only without an effect on Smad4.

TGF-β activity can be downregulated in part by 1) a feedback mechanism involving Smad7, which prevents phosphorylation of Smad2/3 by binding to the TGF-βRI (50, 54), and 2) activation of transcriptional repressor function of Smad6 (43). Our study shows that diabetes is associated with a decrease in renal cortical Smad6 and Smad7 protein expression, indicating that this may be one of the mechanisms by which the profibrotic effect of TGF-β is exerted in the diabetic kidney. Interestingly, the present study demonstrates that E2 supplementation attenuates the diabetes-associated decrease in Smad6 and Smad7 protein expression, suggesting that this may be one of the pathways by which E2 attenuates the profibrotic effects of TGF-β in the diabetic kidney. These observations provide evidence for a dual role for E2 in regulating TGF-β activity in the diabetic kidney: by downregulating the expres-

Fig. 10. Smad6 and Smad7 immunolocalization and protein expression. Top: Smad6 and Smad7 protein expression in representative sections of renal cortex. Original magnification ×400. Bottom: densitometric scans of Smad6 and Smad7 protein levels expressed as ratio of Smad6 to Coomassie blue and ratio of Smad7 to Coomassie blue. Values are means ± SE.
sion of profibrotic signaling molecules (Smad2/3) and by upregulating antifibrotic signaling molecules (Smad6 and Smad7).

In summary, the present study demonstrates that, in the STZ-induced diabetic rat, E2 is renoprotective by attenuating albuminuria and ECM protein expression associated with diabetic glomerulosclerosis and tubulointerstitial fibrosis. One of the mechanisms by which E2 exerts its renoprotective effects in diabetes is regulation of the expression of TGF-β and its downstream signaling pathway involving members of the Smad family of proteins. These findings suggest that E2 supplementation may be beneficial in preventing the development and progression of diabetic renal disease.

ACKNOWLEDGMENTS
The authors acknowledge the technical help of Joseph Garman.

GRANTS
This work was supported by the Carl W. Gottschalk Award from the American Society of Nephrology and a Research Award from the American Diabetes Association to C. Maric.

REFERENCES


