17β-Estradiol replacement improves renal function and pathology associated with diabetic nephropathy

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Mankhey, Richard W., Faizah Bhatti, and Christine Maric. 17β-Estradiol replacement improves renal function and pathology associated with diabetic nephropathy. Am J Physiol Renal Physiol 288: F399–F405, 2005. First published September 28, 2004; doi:10.1152/ajprenal.00195.2004.—The protective factor of female gender appears to be lost in diabetes; the incidence of diabetes and its complications, including diabetic nephropathy, are equal in women and men. This study examined the effects of estrogen deficiency by ovariectomy (OVX) and replacement with 17β-estradiol (OVX+E2) on renal function and pathology in the nondiabetic (ND) and streptozotocin (STZ)-induced diabetic (D) rat kidneys for 12 wk. Diabetes was associated with an increase in urine albumin excretion (UA; ND: 0.39 ± 0.03 D: 5.9 ± 0.8 mg/dky; P < 0.001), decrease in creatinine clearance (CrCl; ND: 0.69 ± 0.03 D: 0.43 ± 0.09 mg·min⁻¹·100 g body wt⁻¹; P < 0.05), increase in the index of glomerulosclerosis [GSI; ND: 0.01 ± 0.01 D: 0.15 ± 0.04 arbitrary units (AU); P < 0.01], tubulointerstitial fibrosis (TIFI; ND: 0.04 ± 0.04 D: 0.68 ± 0.2 AU; P < 0.01), and transforming growth factor-β (TGF-β) protein expression (ND: 0.61 ± 0.02 D: 1.25 ± 0.07 AU; P < 0.01). In the D group, the severity of these changes was augmented with OVX (UA; 8.1 ± 0.6 mg/dky; CrCl; 0.40 ± 0.04 mg·min⁻¹·100 g body wt⁻¹ GSI; 0.29 ± 0.04 AU; TIFI; 0.90 ± 0.06 AU; TGF-β; 1.26 ± 0.10 AU), whereas E2 replacement attenuated these changes (UA; 6.3 ± 0.8 mg/dky; CrCl; 0.66 ± 0.03 mg·min⁻¹·100 g body wt⁻¹ GSI; 0.06 ± 0.02 AU; TIFI; 0.36 ± 0.08 AU; TGF-β; 0.57 ± 0.08 AU). We conclude that E2 deficiency increases the severity of renal disease in a diabetic animal model and that E2 replacement is renoprotective by attenuating the decline in renal function and pathology associated with diabetes.

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Aventis Pharmaceuticals, Kansas City, MO) to maintain blood glucose levels between 250 and 400 mg/dl. During the treatment period (12 wk), the animals (n = 6–8 per group) were placed in metabolic cages every 4 wk and urine samples were collected for measurement of urine albumin excretion (UAE) and CrCl. At the end of the treatment period, the animals were weighed, anesthetized with pentobarbital sodium (40 mg/kg ip), and blood samples were collected, by cardiac puncture, for measurement of plasma levels of E2 and creatinine. Body length was measured for determination of body surface area and body mass index according to the DuBois formula (12). After an abdominal incision was made, the right kidney was removed, and the cortex and medulla were separated and snap-frozen in liquid nitrogen for protein analysis. The left kidney was perfusion fixed with 4% paraformaldehyde for morphological and immunohistochemical analysis. The experiments were performed according to the guidelines recommended by the National Institutes of Health and approved by the Georgetown University Animal Care and Use Committee.

Ovariectomy, estrogen replacement, and plasma estradiol levels. Under gas anesthesia (2% isofluorane), the ovaries were exposed via a midline abdominal incision. This was done to induce a decrease in circulating estradiol levels that are in the peak physiological range in the intact animals (28). After clearing the ovaries from surrounding tissue, the ovarian vessels were ligated with 5-0 silk and the ovaries were removed. In the OVX group, implants of uncoated E2 pellets (10–25% grade 3, affected area 25–75%; grade 4, affected area greater than 75%).

Western blot analysis. Homogenized samples from the renal cortex and medulla were loaded on a 18% SDS-PAGE gel, and the proteins were transferred to a nitrocellulose membrane. The membranes were incubated with 5% nonfat milk, 0.05% Tween 20 in TBS [10 mM Tris·HCl (pH 7.4), 150 mM NaCl], then with the primary antiserum as described for immunohistochemistry at 4°C overnight. The membranes were washed and incubated with goat anti-rabbit 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 following densitometric analysis of gels stained with Coomassie blue. The densities of specific bands were quantitated by densitometry using Scion Image beta (version 4.02) software.

Statistical analysis. Data are expressed as means ± SE and were analyzed with a two-way ANOVA followed by a Bonferroni posttest, using Sigma Stat software. Differences were considered significant at P < 0.05.

RESULTS

Body and kidney weight, body mass index, and food intake. In the ND group, OVX was associated with a 20% increase in body weight, 36% increase in kidney weight, and 14% increase in body mass index (BMI), compared with the intact group, whereas replacement with E2 completely prevented the increases in all three of these parameters; in fact, all three parameters were lower in the OVX+E2 group compared with the intact rats (Table 1). No differences in food intake were observed between the intact, OVX, or OVX+E2 in the ND (Table 1). In the D group, OVX was associated with a more modest (9%) increase in body weight, which was similar to the ND group, whereas E2 replacement decreased body weight to levels similar to the intact animals (Table 1). However, no significant differences in kidney weight or BMI were observed in the D group between the intact and OVX or OVX+E2 animals. Similar to the ND group, no differences in food intake were observed between the intact, OVX, or OVX+E2 in the D (Table 1).

Blood glucose and plasma E2 levels. In the intact D rats, a 390% increase in blood glucose levels was observed compared with the intact ND rats (Table 1). A similar increase in blood glucose was observed in the D group with OVX and OVX+E2 compared with the respective ND group. Plasma estradiol levels were reduced by 53% in the intact D compared with

Table 1. Effects of OVX and E2 replacement on metabolic parameters in diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>ND Intact</th>
<th>D</th>
<th>ND OVX</th>
<th>ND OVX+E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>289±6.9</td>
<td>278±8.2</td>
<td>345±13.1b</td>
<td>301±7.2ab</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.28±0.01</td>
<td>0.27±0.01</td>
<td>0.38±0.03b</td>
<td>0.29±0.01a</td>
</tr>
<tr>
<td>BMI</td>
<td>342.5±5.5</td>
<td>331±3.9</td>
<td>390±3.2</td>
<td>341±5.4a</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>102±2.4</td>
<td>403±22.6a</td>
<td>101±5.6</td>
<td>351±30.4ab</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>17.5±1.9</td>
<td>23.5±2.2a</td>
<td>19.5±0.7</td>
<td>203±2.2</td>
</tr>
<tr>
<td>Plasma E2, pg/ml</td>
<td>40.5±3.5</td>
<td>26.4±7.1a</td>
<td>11.1±0.9ab</td>
<td>9.9±2.1b</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. aP < 0.05, aP < 0.01 vs. nondiabetic (ND) in the same treatment group. bP < 0.05, bP < 0.01 vs. intact in the same metabolic group. cP < 0.05, cP < 0.01 vs. ovariectomy (OVX) in the same metabolic group. D, diabetic; BMI, body mass index.
Table 2. Effects of OVX and E2 replacement on renal and systemic function in diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Intact</th>
<th>OVX</th>
<th>OVX + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ND</td>
<td>D</td>
<td>ND</td>
</tr>
<tr>
<td>UAE, mg/day</td>
<td>0.39 ± 0.03</td>
<td>5.9 ± 0.8&lt;sup&gt;aaa&lt;/sup&gt;</td>
<td>0.67 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CrCl, mg·min⁻¹·100 g body wt⁻¹</td>
<td>0.69 ± 0.03</td>
<td>0.43 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.79 ± 0.12</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>113 ± 3.3</td>
<td>128 ± 8.5</td>
<td>127 ± 8.0</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. UAE, urine albumin excretion; CrCl, creatinine clearance; MAP, mean arterial pressure. *P < 0.05, <sup>aa</sup>P < 0.01, <sup>aaa</sup>P < 0.001 vs. ND in the same treatment group. <sup>b</sup>P < 0.05, <sup>bb</sup>P < 0.01 vs. intact in the same metabolic group. <sup>c</sup>P < 0.05, <sup>cc</sup>P < 0.01 vs. OVX in the same metabolic group.

Fig. 1. Morphology of the nondiabetic (ND) and diabetic (D) rat kidney. A: periodic acid-Schiff-stained sections of the renal cortex. The diabetic kidneys are characterized by moderate glomerulosclerosis (g) with thickening of glomerular basement membrane and mesangium expansion (arrows). The severity of lesions was most pronounced in the ovariectomy (OVX) group, whereas replacement with E2 attenuated these changes. B: Masson’s trichrome-stained sections of the renal medulla. The diabetic kidneys are characterized by moderate tubulointerstitial fibrosis (TIF), characterized by the presence of extracellular matrix deposits within and surrounding the tubulovascular bundles (outlined). The severity of lesions was most pronounced in the OVX group, whereas replacement with E2 attenuated these changes. Original magnification ×400.
intact ND animals (Table 1). In both the ND and D animals, OVX reduced and OVX + E₂ increased plasma estradiol levels to a similar extent.

**UAE, CrCl, and mean arterial pressure.** In the intact D rats, a 1,400% increase in UAE was observed compared with the intact ND rats (Table 2). A similar increase in UAE was observed in the D group with OVX and OVX + E₂ compared with the respective ND group. In both the ND and D groups, the increase in UAE was greatest in the OVX groups compared with intact, whereas E₂ replacement attenuated this increase. In the intact D rats, a 61% decrease in CrCl was observed compared with the intact ND rats (Table 2). Although a similar decrease in CrCl was observed in the D group with OVX compared with the ND OVX group, in the ND group, OVX + E₂ increased CrCl to 53% above that of the D intact group. No differences in mean arterial pressure (MAP) were observed between any of the treatment groups (Table 2).

**GSI and TIFI.** In the ND group, OVX was associated with a ninefold increase in the GSI (Figs. 1 and 2A) compared with the intact group. These kidneys were characterized by glomerular basement thickening and mesangial expansion. Replacement with E₂ attenuated glomerulosclerosis associated with OVX in the ND group and, as observed in the intact group, these kidneys exhibited normal renal morphology (Figs. 1 and 2A). In the D group, the kidneys of intact animals exhibited a 14.5-fold increase in the GSI compared with ND intact animals. As in the ND group, E₂ replacement attenuated these changes (Figs. 1 and 2A). In the ND group, OVX was associated with a ninefold increase in the degree of medullary TIFI (Figs. 1 and 2B) compared with the intact group. These kidneys were characterized by accumulation of ECM deposits in the tubulovascular bundles. Replacement with E₂ attenuated the TIFI associated with OVX in the ND group and as observed in the intact group, these kidneys exhibited normal medullary morphology (Figs. 1 and 2B). In both the renal cortex and medulla in the D group, the kidneys of intact animals exhibited a 6.0- and 17.0-fold increase in the TIFI compared with ND intact animals (Figs. 1 and 2B). As in the ND group, E₂ replacement attenuated these changes (Figs. 1 and 2B).

**TGF-β localization and expression.** In the kidneys of ND rats, no immunostaining for TGF-β could be detected (Fig. 3). In the renal cortex of intact D rats, TGF-β immunostaining was confined to the glomerular mesangium (Fig. 3), whereas in the OVX D group, staining was also prominent in the tubulointerstitium, most noticeably in the proximal tubules. Replacement with E₂ in the D group reduced the intensity of immunostaining and was detectable only in a few glomeruli. Western analysis showed a twofold increase in TGF-β protein expression in the renal cortex of D intact and OVX rats (Fig. 4). E₂ replacement completely prevented the D-induced increase in TGF-β protein expression; the levels of TGF-β protein expression were indistinguishable from the intact ND and D groups.

**DISCUSSION**

In the present study, we demonstrate that in the STZ-induced D rat, replacement with E₂ for 12 wk is renoprotective, by reducing albuminuria, improving CrCl, attenuating glomerulosclerosis and tubulointerstitial fibrosis, and reducing TGF-β protein expression.

Increased UAE and a decline in CrCl are hallmarks of diabetic renal disease (2, 38, 42a). In our study, increased UAE and decreased CrCl were observed after 12 wk of diabetes. The changes in UAE were most pronounced in the OVX group and attenuated by E₂ replacement, whereas changes in CrCl were not as consistent with different treatments within the same metabolic group.

**Fig. 2.** Indexes of glomerulosclerosis (GSI) and TIF (TIFI) in the ND and D rat kidney. A: intact animals in the diabetic group exhibited a 14.5-fold increase in the degree of GSI compared with the intact ND group. OVX exacerbated, whereas E₂ replacement attenuated these changes. B: intact animals in the diabetic cortex and medulla exhibited a 6.4- and 17-fold increase, respectively, in the degree of TIFI compared with the renal cortex and medulla in the intact ND group. OVX exacerbated, whereas E₂ replacement attenuated these changes. Data are expressed as means ± SE; n = 6. *P < 0.05, **P < 0.01 vs. intact in the same metabolic group. a,b P < 0.05 vs. intact in the same metabolic group.
metabolic group. Variable effects of E2 replacement on UAE and CrCl have been reported in other studies. Although in the OLETF diabetic rat E2 had no effect on albuminuria (40), previous studies from this and other laboratories reported that replacement with E2 attenuates the decline in CrCl associated with aging in the Dahl salt-sensitive rat (25) and attenuates albuminuria in the uninephrectomized spontaneously hypertensive rat (18). Furthermore, in type II diabetic patients, replacement with E2 reduced proteinuria and improved CrCl (39). These findings indicate that E2 may prevent as well as reverse the decline in renal function associated with diabetic nephropathy.

At 12 wk of age, the STZ-D rat exhibited moderate glomerulosclerosis, characterized by mild thickening of glomerular basement membrane and mesangial expansion. The degree of glomerulosclerosis was worsened in the absence of ovarian hormones in both the nondiabetic and the diabetic kidney, whereas the damage was attenuated by E2 replacement. These changes in glomerulosclerosis correlated with the changes in UAE. Previous studies reported variable effects of estrogen deficiency and modulation of estrogen effects in experimental models of diabetic nephropathy. In the glomerulosclerosis-prone mouse model with a mutation in oligosyndactyly (ROP Os/+), estrogen deficiency accelerates the progression of glomerulosclerosis (13). In contrast, in the Cohen diabetic rat, estrogen deficiency and treatment with tamoxifen, a selective estrogen receptor modulator, decrease glomerulosclerosis (9, 32). These variable findings are most likely due to different models of diabetic nephropathy, the type of diet fed, as well as the duration of treatments.

Our study suggests that changes in TGF-β protein expression correlate with the degree of glomerular structural damage. Numerous studies have demonstrated that TGF-β promotes renal cell growth and ECM accumulation, the two hallmarks of diabetic nephropathy (8, 17). Although our study did not directly examine the effects of E2 replacement on TGF-β expression, the fact that TGF-β expression decreased in animals replaced with E2 suggests that one of the mechanisms by which E2 is renoprotective may be through regulating TGF-β expression. Estradiol and its metabolites have been shown to inhibit TGF-β protein expression under hyperglycemic conditions (26, 28). Further studies are needed to examine the effects of E2 on regulating TGF-β expression in our experimental model.

In addition to glomerulosclerosis, the STZ-D rat also exhibited moderate TIF in both the cortex and medulla, character-
ized by accumulation of ECM deposits in the tubulovascular bundles, presence of inflammatory infiltrates and tubular casts. Most recent studies have suggested that the degree of tubulointerstitial fibrosis is the most reliable marker of the progression of renal disease (16). The mechanisms leading to these changes have not been explored in the current study, but numerous other studies demonstrate local activation of growth factors and cytokines, including angiotensin, endothelin, and TGF-β in response to hyperglycemia (8, 16, 42). Similar to glomerulosclerosis, we found that the degree of tubulointerstitial fibrosis correlated with the changes in albuminuria and that E2 replacement attenuated these changes. A similar renoprotective effect of E2 has been observed in the 5/6 remnant kidney model, in which E2 replacement inhibited tubulointerstitial fibrosis via reducing TGF-β expression (4, 22). In the ROP Os/+ mice, E2 also prevented tubulointerstitial fibrosis by decreasing synthesis of interstitial and basement membrane collagens (13), whereas in cultured mesangial cells, E2 increases the activity of ECM-degrading enzymes, matrix metalloproteinases (30), and inhibits TGF-β-induced apoptosis (27). Similar mechanisms may underlie the renoprotective effect of E2 in our model, and further studies are underway to more specifically examine these mechanisms.

Although several studies suggest that the renoprotective effects of E2 are mediated via its blood pressure-lowering effects (11, 39), our studies show that E2 is renoprotective irrespective of its effect on blood pressure. No differences in MAP were observed in any of the treatment groups, suggesting that the renoprotective effects of E2 may indeed be direct, most likely via mediating cellular process such as cell proliferation and ECM metabolism.

Estrogen-deficient animals, in both D and ND states, exhibited an increase in body weight and BMI. These findings are in concert with the previously reported studies on the increase in body weight and BMI associated with menopause and decline in ovarian function. Although the mechanisms for the increase in body weight and BMI in the absence of ovarian hormones are not completely understood, a recent study showed that the increase is not due to an increase in food intake but rather due to altered levels of luteinizing hormone and leptin (1, 23). Similar mechanisms may be responsible for the prevention of body weight gain after E2 replacement in the STZ-D rat, as no differences in food intake were observed between intact, OVX, and OVX+E2 rats.

An interesting observation from our studies is that the STZ-induced D rat has lower circulating estradiol levels compared with its ND counterpart. Although very few studies in fact report the hormone levels in patients with diabetic nephropathy, there are some reports that suggest that diabetic women also have lower circulating levels of estradiol (31). These studies point to a relationship between ovarian hormones and diabetes and this interaction may contribute to the mechanisms underlying why women with diabetes “lose” the protective effect of the female sex and exhibit prevalence and progression of disease that are similar to men. Our findings suggest that E2 replacement may be beneficial in preventing or retarding the development of diabetic renal complications. Interestingly, however, our studies also indicate that despite the similar levels of E2 in the intact and E2-replaced group with diabetes, the E2-replaced group is characterized by higher CrCl and lower markers of renal pathology compared with the intact. These findings suggest that the presence of progesterone in the intact rats (the E2-replaced animals are deficient of progesterone as a result of ovariectomy) may have adverse effects in the face of hyperglycemia. Although no studies to date have addressed the effects of progesterone in diabetic nephropathy, a study examining the effects of E2 alone and combined E2 and progesterone in type II diabetic women reported beneficial effects of E2 alone, but not the combined study (37). These findings suggest that the presence of progesterone may attenuate the beneficial effects of E2 in diabetes. Further studies are warranted to examine the specific effects of progesterone in diabetic renal complications.

Despite the early termination of the E2 alone arm of the Women’s Health Initiative (WHI) trial due to adverse effects of conjugated equine estrogen on stroke and hip fractures, this study did not show adverse effects on cardiovascular, whereas the renal effects have not even been examined in this study (3). Furthermore, the WHI trial was performed on average in woman who had been postmenopausal for at least 10 years, that is women who have experienced low levels of ovarian hormones for an extended period of time. Our animal studies were performed in young, premenopausal animals to examine if E2 replacement attenuates the development of diabetic nephropathy in animals that have not experienced an age-related decline in circulating estradiol. Whether E2 is also beneficial in attenuating or reversing the disease process once it has advanced or after the animals have been estrogen depleted for an extended period of time, such as with aging, remains to be elucidated.

In summary, our study demonstrates that E2 in the STZ-induced D rat is renoprotective by preventing the decline in renal function and pathological changes associated with diabetic nephropathy. These findings suggest that E2 replacement may be beneficial in preventing diabetic renal complications, but most importantly, stress the importance of examining the contribution of gender and sex hormones in the incidence and progression of cardiovascular disease, renal disease, and diabetes.

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