Glomerulosclerosis and Tubulointerstitial Fibrosis are Attenuated with 17β-Estradiol in the Aging Dahl Salt Sensitive Rat

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Abstract. This study examined the effects of estrogen deficiency by ovariectomy (OVX) and 17β-estradiol (E2) replacement (OVX+E2) on glomerulosclerosis and tubulointerstitial fibrosis and the mechanisms contributing to these changes, including expression of collagen type IV and laminin, transforming growth factor-β (TGF-β), and activity of matrix metalloproteinases (MMP) in the kidneys of young (4 mo [4M]) and aged (12 mo [12M]) Dahl salt-sensitive (DSS) rats maintained on a low-salt (0.1% NaCl) diet. While normal renal morphology was observed in the 4M rats in all treatment groups, moderate to severe glomerulosclerosis (glomerulosclerotic index [GSI]: 4M, 0.22 ± 0.09 versus 12M, 1.43 ± 0.17; P < 0.001) and cortical tubulointerstitial fibrosis (CTIFI: 4M, 0 versus 12M, 57.1 ± 4.9; P < 0.01) was observed in the 12M rats. The severity of glomerulosclerosis and cortical tubulointerstitial fibrosis in the 12M group was augmented with OVX (GSI, 3.27 ± 0.34; CTIFI, 74.4 ± 9.2; P < 0.01 versus intact at 12M) and attenuated with E2 replacement (GSI, 1.09 ± 0.09; CTIFI, 49.2 ± 6.8). In the 12M animals, OVX was also associated with increased deposition and expression of laminin (Intact, 228.1 ± 6.7; OVX, 277.4 ± 9.6 AU; P < 0.01), increased expression of TGF-β (Intact, 85.0 ± 23.0; OVX, 178.0 ± 20.5 AU; P < 0.001), and decreased activity of cortical MMP-9 (Intact, 3.8 ± 0.8; OVX, 2.4 ± 0.6 AUC; P < 0.01). E2 replacement opposed these effects (laminin, 229.9 ± 6.2 AU; TGF-β, 101.3 ± 25.2 AU; MMP-9, 5.2 ± 0.2 AUC). The severity of the disease in the 12M rats correlated with a modest decrease in creatinine clearance (Intact, 0.26 ± 0.01; OVX, 0.22 ± 0.01; OVX+E2, 0.28 ± 0.01 mg/min per 100 g) and increase in BUN (Intact, 20.3 ± 2.1; OVX, 32.6 ± 5.1; OVX+E2, 24.3 ± 2.4 mg/dl). The authors conclude that E2 is renoprotective in the aging DSS rat by attenuating glomerulosclerosis and tubulointerstitial fibrosis.

The rate of progression of cardiovascular and renal disease and hypertension increases with age and is lower in premenopausal women compared with age-matched men (1–3). Interestingly, a re-evaluation of the Modification of Diet in Renal Disease (MDRD) study showed that the differences in the rate of decline of renal function between men and women is reduced after age 52 yr (their dividing point) (4), suggesting that menopause may affect the progression of renal disease in women; however, no studies to date have clearly demonstrated this point, suggesting that our understanding of the contribution of ovarian hormones to the development of age-related disease processes remains unclear. The Women’s Health Initiative (WHI) reported that hormone replacement therapy with the estrogen-progestrone combination Prempro (conjugated equine estrogen and progestin) causes a slight increase in the risk of cardiovascular disease in a large population of postmenopausal women (5,6). However, numerous studies indicate that estrogen alone has cardiovascular protective effects. Estrogen improves serum lipid levels, increases endothelium-dependent vasodilation and markers of fibrinolysis and vascular inflammation, and protects against the development of atherosclerosis and hypertension (3,5,7–9). Even though the estrogen-alone arm of the WHI trial has recently been terminated (10) (partly because estrogen did not appear to affect heart disease, which was the main question of the study), no renal effects have been evaluated in these trials. The controversy surrounding the question of whether or not estrogen is protective against disease processes indicates the need for further understanding the role estrogen plays in the development and progression of disease processes associated with aging.

Progressive deterioration of renal structure and function occurs with aging. The aging kidney is characterized by a decrease in GFR and renal blood flow (11–13), decreased urine-concentrating ability (14), increased responsiveness to angiotensin II and endothelin (15–17), decreased production of vasodilatory prostaglandins and nitric oxide (18,19), increased oxidative stress (20,21), and increased glomerulosclerosis and...
Few studies, however, have investigated the role of E2 on the aging kidney. In this study, we examined the effects of E2 on glomerulosclerosis and tubulointerstitial fibrosis in a rat model of age-related renal disease. We tested the hypothesis that estrogen loss would accelerate, while estrogen replacement would limit the progression of age-related renal disease.

Materials and Methods

Animal Model

Dahl salt-sensitive (DSS) rats (Rapp Strain; Harlan Sprague Dawley, Madison, WI) were purchased at 6 to 7 wk of age and maintained on a low (0.1% NaCl) phytoestrogen-free rat chow diet (Harlan Teklad), and given water ad libitum. At 3 mo of age, the animals (n = 4 to 6 per treatment group) were randomly divided and subjected to sham surgery (Intact), ovariectomy (OVX), and OVX with implantation of an E2 silastic pellet (OVX+E2), according to the methods described below. The treatments were carried out for either 1 or 9 mo, rendering the animals 4-mo-old (4M) or 12-mo-old (12M) at the time described below. The treatments were carried out for either 1 or 9 mo, rendering the animals 4-mo-old (4M) or 12-mo-old (12M) at the time of sacrifice, respectively. The animals were sacrificed by decapitation, and plasma samples were collected for measurement of E2 levels. The right kidney was removed and sacrificed in liquid nitrogen (for Western blot analysis). The experiments were performed according to the guidelines recommended by the National Institutes of Health and approved by the University of Texas Health Science Center and Georgetown University Animal Care and Use Committees.

OVX, Estrogen Replacement, and Plasma Estradiol Levels

Under gas anesthesia (2% isofluorane), the ovaries were exposed via bilateral flank incisions and excised. The animals receiving E2 replacement therapy were implanted with E2 (Sigma, 5 mg)–filled silastic tubes as described previously (28,29). This dose of E2 yields circulating estradiol levels that are in the peak physiologic range. The pellets were removed and replaced every 12 wk. Sham operations were carried out in the third group of animals in which only skin incisions were made without excising the ovaries.

At the time of sacrifice, uterine tissue was weighed and plasma collected for measurement of estradiol using the commercially available kit (Diagnostic Systems Lab., Webster, TX) according to the manufacturer’s protocol.

Creatinine Clearance and Plasma BUN

Plasma and urine creatinine concentrations were determined using the modified Jaffe rate method (31). Plasma blood urea nitrogen (BUN) levels were measured using the UV rate method (32). Urine samples were analyzed with a Ciba-Corning Express-Plus Analyzer (Polstar Laboratories, Escondido, CA). Plasma samples were analyzed with a Beckman Synchrone CX5CE Analyzer.

Morphology

After fixation in 4% paraformaldehyde, the tissue was processed to paraffin, sectioned at 4 μm, and stained with periodic acid Schiff (PAS, for demonstration of glycerogen deposits) or Masson trichrome (for demonstration of collagen deposition) (33).

Glomerulosclerotic Index

PAS-stained sections were examined using a Nikon Eclipse E600 light microscope. One hundred glomeruli per section were randomly selected and the degree of glomerular damage assessed using a semi-quantitative scoring method: grade 0, normal glomeruli; grade 1, sclerotic area up to 25% (minimal sclerosis); grade 2, sclerotic area 25 to 50% (moderate sclerosis); grade 3, sclerotic area 50 to 75% (moderate-severe sclerosis); grade 4, sclerotic area 75 to 100% (severe sclerosis). The glomerulosclerotic index (GSI) was calculated using the following formula: GSI = (3/n4) + (2/n3) + (3/n3) + (4/n1)/n0 + n1 + n2 + n3 + n4, where n is the number of glomeruli in each grade of glomerulosclerosis (34). This analysis was performed with the observer masked to the treatment groups.

Assessment of Tubulointerstitial Fibrosis

Masson trichrome-stained sections were examined using a light microscope. The degree of tubulointerstitial fibrosis was defined as tubular atrophy or dilatation, presence of inflammatory cells, deposition of ECM, and interstitial cell proliferation. The degree of tubulointerstitial fibrosis was graded on a scale of 0 to 4: grade 0, affected area 0% (normal); grade 1, affected area less than 10%; grade 2, affected area 10 to 25%; grade 3, affected area 25 to 75%; grade 4, affected area greater than 75%. Estimation of tubulointerstitial fibrosis was performed with the observer masked to the treatment groups.

Immunohistochemistry

Paraffin sections (4 μm) were incubated with antisera against collagen type IV (Southern Biotech, AL), laminin (Sigma), and TGF-β (R&D Systems, MN) at 4°C overnight. The sections were rinsed with phosphate-buffered saline then incubated with either mouse or rabbit biotinylated IgG, then with avidin-biotin complex (Vectastain ABC kit). Positive immunoreaction was identified after incubation with 3’-3’-diaminobenzidine tetrahydrochloride dihydrate and counterstaining with Mayer hematoxylin. Sections incubated with 10% non-immune goat serum instead of the primary antiserum were used as negative controls.

Western Blotting

The renal cortex and medulla were separated and homogenized in a 40 mM Tris (pH 7.3) containing 20 μM leupeptin, 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 01% Triton-X. The protein concentration of the samples was determined using a colorimetric assay (BioRad, Hercules, CA) with bovine serum albumin as a standard. The samples (containing 40 μg of protein) were denatured in a buffer containing 62.5 mM Tris HCl, 2% SDS, 10% glycerol, 0.004% bromophenol blue, and 5% β-mercaptoethanol, for
5 min at 95°C, separated on a 12% acrylamide gel at 200 V, and electroblotted to nitrocellulose membranes at 250 mA. Following incubation with the primary antisera to laminin and TGF-β, immunoblotting was performed using the Lumiglow kit (KPL, Gaithersburg, MD) following the manufacturers protocol. The levels of protein expression were quantitated by densitometry using the Scion image beta (version 4.02) software. The densities of the specific bands were adjusted to total amount of protein loaded following densitometric analysis of gels stained with Coomassie blue.

Zymography

Cortical and medullary 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 (BioRad). Gelatinolytic activity of matrix metalloproteinase-2 (MMP-2) and MMP-9 was visualized as clear bands against a blue background following staining with Coomassie blue. Bands were quantitated by densitometry using Scion image beta (version 4.02) software.

Statistical Analyses

Data are expressed as means ± SEM and were analyzed using a two-way ANOVA followed by a Newman-Keuls multiple comparison test. Differences were considered statistically significant at P < 0.05.

Results

Body, Kidney, and Uterine Weight and Plasma Estradiol Levels

In comparison to Intact animals at 4M, body weights in the OVX group were increased by 1.2-fold, while body weights in the E2-replaced group were reduced by 1.1-fold (Table 1). In the 12M animals, body weights in the OVX group were also increased by 1.1-fold when compared with the Intact group; however, body weights of the E2-replaced group, while lower than the OVX animals, were not statistically different from the Intact group. No differences in kidney weight were observed between the animals at 4M (Table 1). At 12M, an increase in kidney weight was observed in the E2-replaced group compared with both the Intact and OVX groups.

Plasma estradiol levels declined with age in the Intact animals and were sevenfold lower at 12M when compared with 4M. Compared with the 4M group, the 12M OVX animals had slightly lower circulating estradiol levels, and the levels were significantly reduced when compared with Intact animals of the same age. At 4M, E2 replacement produced estradiol levels, which were indistinguishable from the Intact animal group, while E2 levels in the 12M E2 replacement group were 3.5-fold higher compared with Intact animals (Table 1).

The Intact 12M rats had a 1.3-fold lower uterine weight compared with 4M (Table 1). Atrophy of uterine tissue was observed in 4M and 12M with OVX, while replacement with E2 was associated with similar uterine weights as Intact rats in both the 4M and 12M animals.

Creatinine Clearance and BUN

No differences in creatinine clearance (CrCl) or BUN levels were observed between treatment groups in the 4M animals. An overall decrease in CrCl and an increase in BUN levels were observed in the 12M compared with 4M animals. In the 12M animals, a modest, 1.1-fold decrease in CrCl and 1.3-fold increase in BUN were observed with OVX compared with Intact, which was prevented with E2 replacement.

Glomerulosclerosis

The kidneys of 4M DSS rats, whether intact, OVX, or OVX+E2, exhibited normal glomerular morphology (Figure 1, A-C). In the 12M DSS animals, there was an overall increase in the degree of glomerulosclerosis compared with 4M in all

Table 1. Body and kidney weight, creatinine clearance (CrCl), blood urea nitrogen (BUN) and plasma estradiol in 4-mo-old (4M) and 12-mo-old (12M) Dahl salt-sensitive (DSS) rats

<table>
<thead>
<tr>
<th></th>
<th>Intact 4M</th>
<th>Intact 12M</th>
<th>OVX 4M</th>
<th>OVX 12M</th>
<th>OVX + E2 4M</th>
<th>OVX + E2 12M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>262.5 ± 9.4</td>
<td>377.7 ± 17.9 ^aa</td>
<td>317.3 ± 5.2 ^a</td>
<td>421.0 ± 18.0 ^bb</td>
<td>228.3 ± 2.3 ^aa ^ccc</td>
<td>285.8 ± 9.3 ^bb ^ccc</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.88 ± 0.02</td>
<td>1.11 ± 0.04</td>
<td>0.83 ± 0.02</td>
<td>1.03 ± 0.04</td>
<td>0.93 ± 0.03</td>
<td>1.4 ± 0.11 ^dd ^ccc</td>
</tr>
<tr>
<td>Uterine weight (mg)</td>
<td>434.0 ± 34.0</td>
<td>344.9 ± 25.8 ^aa</td>
<td>97.0 ± 3.0 ^aa</td>
<td>156.0 ± 11.1 ^bb</td>
<td>394.5 ± 13.6 ^ccc</td>
<td>379.6 ± 41.4 ^ddd</td>
</tr>
<tr>
<td>CrCl (ml/min/100g)</td>
<td>0.33 ± 0.01</td>
<td>0.26 ± 0.01 ^a</td>
<td>0.29 ± 0.02</td>
<td>0.22 ± 0.03 ^c</td>
<td>0.37 ± 0.01 ^e</td>
<td>0.28 ± 0.01 ^dd</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>24.7 ± 1.5</td>
<td>20.3 ± 2.1</td>
<td>18.8 ± 3.1</td>
<td>32.6 ± 5.1 ^bb ^ccc</td>
<td>20.0 ± 1.5</td>
<td>24.3 ± 2.4 ^dd</td>
</tr>
<tr>
<td>Plasma estradiol (pg/ml)</td>
<td>38.1 ± 9.4</td>
<td>5.5 ± 1.3 ^aa</td>
<td>12.7 ± 1.5 ^aa</td>
<td>6.6 ± 1.5 ^c</td>
<td>32.5 ± 2.4 ^cc</td>
<td>19.4 ± 2.6 ^ddd ^ccc</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. ^aP < 0.05, ^aaP < 0.01, ^aaaP < 0.001 versus Intact at 4M; ^bP < 0.05, ^bbP < 0.01, ^bbbP < 0.001 versus Intact at 12M; ^cP < 0.05, ^ccP < 0.01, ^cccP < 0.001 versus OVX at 4M; ^dP < 0.05, ^ddP < 0.01, ^dddP < 0.001 versus OVX at 12M; ^eP < 0.05, ^eeP < 0.01, ^eeeP < 0.001 versus OVX + E2 at 4M.
animal groups. The kidneys of the 12M Intact group exhibited moderate glomerulosclerosis (Figure 1D, Table 2). Both diffuse and nodular glomerulosclerosis was observed, which was characterized by glomerular basement membrane thickening, mesangial expansion, and the occasional presence of nodules resembling Kimmelstiel-Wilson nodules. The degree of glomerulosclerosis was 2.3-fold greater in the OVX group compared with Intact (Figure 1E, Table 2), while with E$_2$ replacement, the degree of glomerulosclerosis was similar to the Intact group (Figure 1F, Table 2).

**Tubulointerstitial Fibrosis**

The kidneys of 4M rats, whether intact, OVX, or OVX+E$_2$, exhibited normal tubulointerstitial morphology in the cortex (Figure 1, A-C) and medulla (Figure 2, A-C). At 12M, there was an overall increase in the degree of tubulointerstitial fibrosis both in the cortex and medulla compared with 4M in all treatment groups. The kidneys of the Intact group at 12M exhibited moderate tubulointerstitial fibrosis (Figures 1D and 2D, Table 2). These changes were characterized by accumulation of ECM, prominent inflammatory infiltrates, tubular dilatation, tubular atrophy, and the presence of tubular casts, which were more prominent in the medulla than the cortex. At 12M, the degree of tubulointerstitial fibrosis in the cortex was 1.3-fold greater in the OVX group compared with Intact animals, whereas the degree was 12-fold greater in the medulla (Figures 1E and 2E, Table 2). $E_2$ replacement protected against the age-associated pathology that developed in both the cortex and medulla of the 12M OVX animal, and the degree of tubulointerstitial fibrosis was not distinguishably different from the Intact group (Figures 1F and 2F, Table 2).

**Collagen Type IV and Laminin Deposition**

Collagen type IV (Figure 3) and laminin (Figure 4) were immunolocalized to the basement membranes in all epithelial structures in both the renal cortex and medulla. In 4M rats, there were no differences in the pattern of immunolocalization of either collagen type IV or laminin with OVX or with E$_2$ replacement compared with the Intact group. In 12M rats, there was an apparent increase in both collagen type IV and laminin immunostaining in the GBM as well as in the mesangium (Figures 3, D-F, and 4, D-F); this increase was more apparent in the OVX group; with $E_2$ replacement, the pattern of immunostaining resembled that observed in the Intact group. Western analysis revealed an overall increase in laminin protein content in the renal cortex of 12M compared with 4M rats in all treatment groups (Figure 5). The largest increase (1.2-fold) in laminin protein content was observed in the OVX group when compared with Intact, and laminin levels were not distinguishable between the Intact and E$_2$-replaced groups at 12M (Figure 5).

**Transforming Growth Factor-β Localization**

No immunostaining for transforming growth factor-β (TGF-β) could be detected in 4M rats (Figure 6, A-C). In 12M rats, TGF-β immunostaining was observed in the glomerular mesangium (Figure 6D). Immunostaining was more intense in the OVX group, in which, in addition to the mesangium,
Table 2. Glomerulosclerosis and tubulointerstitial fibrosis in 4M and 12M DSS rats

<table>
<thead>
<tr>
<th></th>
<th>Intact</th>
<th>OVX</th>
<th>E2-replaced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulosclerotic index</td>
<td>0.22 ± 0.09</td>
<td>0.19 ± 0.03</td>
<td>0.27 ± 0.17</td>
</tr>
<tr>
<td>Cortical tubulointerstitial damage index</td>
<td>1.43 ± 0.17</td>
<td>0.31 ± 0.20</td>
<td>0.71 ± 0.49</td>
</tr>
<tr>
<td>Medullary tubulointerstitial damage index</td>
<td>0.22 ± 0.09</td>
<td>0.19 ± 0.03</td>
<td>0.27 ± 0.17</td>
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</table>

Data are expressed as mean ± SEM. P < 0.01, P < 0.001 versus Intact at 4M; b, P < 0.05 versus OVX at 4M; c, P < 0.001 versus OVX at 12M. *P < 0.05 versus OVX at 4M.

MMP Activity

In the renal cortex of 4M rats, the activity of both MMP-2 and MMP-9 was detected by gelatin zymography (Figure 8), although MMP-9 was the predominant isoform. In 12M rats, overall MMP-9 activity was reduced compared with 4M rats in all treatment groups. This reduction was most pronounced in the OVX group, which exhibited a 1.4-fold and 1.3-fold decrease in MMP-9 activity in the cortex and medulla, respectively, compared with the Intact group. With E2 replacement, the activities of both MMP-2 and MMP-9 were similar to the Intact group.

Discussion

In the present study, we demonstrate that DSS rats maintained on a low-salt diet for 12 mo develop glomerulosclerosis and tubulointerstitial fibrosis similarly to the age-related renal changes observed in humans. Moreover, we show that the severity of both glomerulosclerosis and tubulointerstitial fibrosis is most pronounced in the absence of ovarian hormones and that these effects are prevented with E2 replacement.

Our study demonstrates that the 12M DSS rats maintained on a low-salt (0.1% NaCl) diet develop age-related renal pathophysiology within 1 yr of age. Furthermore, the renal pathology that develops in this relatively short period of time is highly similar to that observed in humans (23,35,36). In comparison, the widely used Fischer 344 rat model of aging does not exhibit significant renal pathology even by 24 mo of age (unpublished observation). Therefore, the DSS rat is a valuable animal model for the study of age-related renal disease.

The 12M DSS rat kidney is characterized by moderate to severe glomerulosclerosis and tubulointerstitial fibrosis. Thickening of basement membrane, expansion of the mesangium, and accumulation of collagen type IV and laminin were observed in the glomerulus. Deposition of collagen type IV and laminin were also observed in the tubulointerstitium, where inflammatory infiltrates and presence of tubular casts were prominent. Interestingly, it was not only the renal cortex that was affected by age-related structural changes; the pathology of the renal medulla was just as marked. To date, few studies have examined the contribution of the medullary interstitium to age-related renal disease. The mechanisms leading to pathologies in the medulla may be different in that in the renal cortex, and elucidating these mechanisms may contribute to our understanding of age-related renal disease.

Increased deposition of collagen type IV and laminin, as observed in our study, has previously been shown to be associated with a number of progressive renal diseases, including...
Recent studies have shown that in addition to excessive synthesis of the ECM, the presence of amorphous ECM deposits in progressive renal disease are due to decreased activity of the ECM degradative pathway (38,39). Our studies extend these findings by providing evidence of decreased activity of MMP, specifically MMP-9 and MMP-2, in the 12M kidney, thus contributing to the age-related accumulation of ECM in tubulointerstitial fibrosis and glomerulosclerosis.

Figure 2. PAS-stained sections of the renal medulla of 4M and 12M DSS rats. 4M animals show normal medullary morphology (A-C). The kidneys of 12M Intact animals (D) show tubular dilatation and atrophy, numerous proteinaceous casts (c), accumulation of ECM (arrow head), and the presence of inflammatory infiltrates (arrow). The severity of these changes was most pronounced after OVX (E), while E2 replacement prevented these processes (F). Original magnification, ×400.

Figure 3. Immunohistochemical localization of collagen type IV in kidneys of 4M and 12M DSS rats. In 4M kidneys (A-C), collagen type IV (brown staining) was observed in the basement membranes of proximal tubules (pt), collecting ducts (cd), and glomeruli (g). In the 12M Intact animals (D), the intensity of immunostaining was increased in the basement membranes of all tubules and the glomeruli with increased deposition in the expanded mesangium (arrow heads). This deposition was augmented by OVX (E) and abrogated by E2 treatment (F). Original magnification, ×400.
The primary mechanisms underlying age-related renal disease are unknown, but many studies suggest that increases in local vasoconstrictor and growth-promoting factors and/or decreases in vasodilator and growth-inhibiting factors contribute to these pathologic processes (19,23,40). In the aging DSS kidney, the severity of renal pathology was associated with increased TGF-β immunolocalization and protein expression. Our finding is consistent with previous observations showing that TGF-β is a multifunctional polypeptide growth factor whose expression is enhanced in renal diseases associated with abnormal regulation of ECM metabolism (41,42). TGF-β plays a crucial role in the pathophysiology of glomerulosclerosis and tubulointerstitial fibrosis, and its increased expression is associated with decline in renal function (42); however, the mechanisms that lead to its induction remain unknown. Studies in animal models of diabetic nephropathy report that angiotensin II increases TGF-β mRNA expression and that inhibitors of the renin-angiotensin system prevent this increase (43). Given the similarity in pathology of diabetic and age-related renal disease, it will be interesting in future studies to determine if a similar mechanism regulates TGF-β expression in the aging kidney.

The most striking observation from our studies of the DSS rat is that the severity of the age-related structural damage was markedly increased in the absence of ovarian hormones and that E2 replacement attenuated these increases. The structural damage in the OVX animals was associated with a modest decline in creatinine clearance, increase in BUN, and increase in body weight; these changes were attenuated with E2 replacement. These findings support a renoprotective role for E2 in age-related renal disease.

Although the estrous cycle of DSS animals has not been characterized, our studies indicate that 12M animals have not completely stopped cycling (unpublished observations). This observation is consistent with the low levels of circulating E2 and modestly reduced uterine weights observed in these animals. Although still cycling, these reductions in E2 levels and

Figure 4. Immunohistochemical localization of laminin in kidneys of 4M and 12M DSS rats. In 4M kidneys (A-C), laminin (brown staining) was observed in the basement membranes of proximal tubules (pt), collecting ducts (cd), and glomeruli (g). In the 12M Intact animals (D), the intensity of immunostaining was increased in the basement membranes of all tubules and the glomeruli with increased deposition in the expanded mesangium (arrow heads). This deposition was augmented by OVX (E) and abrogated by E2 treatment (F). Original magnification, ×400.

Figure 5. Western analysis of laminin expression in kidneys of 4M and 12M DSS rats. In the renal cortex of 4M animals, no difference in laminin protein expression was observed among the Intact, OVX, and OVX+E2 groups. In 12M animals, laminin protein expression was increased in the OVX group compared with Intact, while E2 treatment restored this to the levels observed in the Intact animals. Top panel, SDS gels of laminin protein expression; bottom panel, densitometric scans in arbitrary units (AU) of laminin protein from top panel. Data are expressed as mean ± SEM; n = 4.
uterine weight most likely reflect age-related changes. OVX clearly resulted in uterine atrophy and a depletion of circulating E2. This effect of OVX was reversed with E2 replacement therapy, which resulted in uterine weights that were similar to Intact animals and circulating E2 levels that were within the normal physiologic range (30).

The E2 replacement in our study began while the animals were young and estrogen replete. Thus the 12M E2-replaced animals never experienced a decline in reproductive function in contrast to the Intact animals, which experienced a sevenfold decrease in estradiol levels by 12 mo of age even though they had not reached the age of cycle cessation as observed in other rat strains (44). The aim of our study was to examine the effects of OVX and E2 replacement in aged DSS rats that were in the state of E2 deficiency rather than menopause (cessation of cycling). It is worth noting that the WHI trial was conducted in women who were predominantly menopausal before the onset of their hormone replacement treatment, and thus had been exposed to low circulating levels of estradiol. Therefore, our experimental design is significantly different from that of the WHI in that we are investigating the role of estrogen at preventing the development of age-related disease whereas the WHI trial investigated the role of estrogen at reversing the development of age-related disease.

Our study suggests that E2 replacement reduces glomerulosclerosis and tubulointerstitial fibrosis by attenuating the age-related accumulation of collagen type IV and laminin. Furthermore, we show that the activities of MMP-9 and MMP-2 are increased with E2 treatment, suggesting that E2 promotes ECM degradation. Our findings support previous studies, which have reported a similar renoprotective role of estrogen in sclerosis-prone mice (26), in the remnant kidney model (45), and in cultured mesangial cells (27) via similar mechanisms. It will be interesting in future studies to also examine the role of testosterone, as some studies indicate that it is not only the estrogen-deficient state that affects renal disease progression but also the change in ratio of estrogen to testosterone after estrogen deficiency (46). Another potential contributing factor worth investigating in future studies is body weight because...
suggesting that the renoprotective effects of E2 replacement in this animal thus correlate with the changes in renal pathology, to near normotensive levels. The age-related changes in BP in Intact and OVX, while E2 treatment increased MMP-9 activity 12M animals, no differences in MMP-9 activity were observed between Intact and OVX, while E2 treatment restored this to the levels even higher than observed in the Intact animals. In the medulla of 4M and 12M DSS rats. In the renal cortex (A) and medulla (B) of 4M (MMP-2) and MMP-9 activity in the renal cortex and medulla in 4M processed from top panel. Data are expressed as mean ± SEM; n = 4.

Figure 8. Zymographic analysis of matrix metalloproteinase–2 (MMP-2) and MMP-9 activity in the renal cortex and medulla in 4M and 12M DSS rats. In the renal cortex (A) and medulla (B) of 4M animals, no differences in MMP-9 activity was observed. In the cortex of 12M animals (A), MMP-9 activity was reduced in the OVX group compared with Intact, while E2 treatment restored this to the levels even higher than observed in the Intact animals. In the medulla of 12M animals, no differences in MMP-9 activity were observed between Intact and OVX, while E2 treatment increased MMP-9 activity compared to both the Intact and OVX group. Top panel in A and B, Zymograms gels of MMP-9 and MMP-2 activity. Bottom panel, Densitometric scans in arbitrary units (AU) of MMP-9 zymograms from top panel. Data are expressed as mean ± SEM; n = 4.

body weights were increased in the OVX animal group compared with Intact and estrogen-replaced animals as found by other investigators (47,48).

Salt sensitive hypertension is a condition that affects over 30% of postmenopausal women (49). Previous reports in DSS rats indicate that there is a correlation between the severity of renal function and hypertension in animals lacking ovarian hormones (50,51). Furthermore, Hinojosa-Laborde et al. (52) have reported that the DSS rat develops mild hypertension with aging despite being maintained on a low-salt diet; OVX further exacerbates the hypertension, while E2 replacement lowers BP to near normotensive levels. The age-related changes in BP in this animal thus correlate with the changes in renal pathology, suggesting that the renoprotective effects of E2 replacement may be mediated via its effects on BP. However, several studies suggest that estrogens exert a renoprotective effect independently of their BP-lowering effects (3,26) by reducing cell death by apoptosis and increasing ECM degradation. Studies in this laboratory have shown that, in a STZ model of diabetes, E2 replacement reduces renal collagen type IV synthesis and tubulointerstitial fibrosis without an effect on BP (53). Thus it is likely that renoprotective effects of estrogen are mediated directly, via regulating cell growth and ECM metabolism, and indirectly, via its effect on BP.

In summary, the present study demonstrates that 12M DSS rats develop glomerulosclerosis and tubulointerstitial fibrosis and a decline in creatinine clearance that is most severe in the absence of ovarian hormones and is attenuated with E2 replacement. Therefore, this study suggests that E2 replacement therapy may be beneficial in limiting the progression of age-related renal disease and that the aging DSS rat is a valuable model for investigating age-related kidney disease.

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