Beneficial Effects of Estrogens on Indices of Renal Damage in Uninephrectomized SHRsp Rats

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Abstract. Renal diseases tend to be less severe among premenopausal female patients, compared with male patients. Experimental data on the effects of estrogens on renal damage are controversial, and potential underlying mechanisms have not been fully clarified. Three-month-old, female, uninephrectomized (UNX), sham-operated or ovariectomized (OVX) SHRsp rats were left untreated or received either 17β-estradiol 3-benzoate (25 μg/d) or estriol (0.02 mg/d) daily. After 3 mo, indices of renal damage (glomerulosclerosis index and tubulointerstitial damage index) and glomerular geometric parameters were investigated. The expression of desmin, TGF-β, endothelin-1, collagen IV, endothelial nitric oxide synthase, and estrogen receptors α and β in the glomeruli and tubulointerstitium was immunohistochemically evaluated. Estradiol and estriol did not significantly affect kidney weights or BP. Estradiol and estriol caused significant reductions in albuminuria (vehicle-treated UNX/OVX animals, 25.4 $\pm$ 8.52 mg/24 h; estradiol-treated UNX/OVX animals, 15.37 $\pm$ 6.12 mg/24 h; estriol-treated UNX/OVX animals, 6.54 $\pm$ 2.24 mg/24 h). The glomerulosclerosis index was significantly lower in estriol- and estradiol-treated animals (estradiol-treated UNX/OVX animals, 0.69 $\pm$ 0.16; estriol-treated UNX/OVX animals, 0.21 $\pm$ 0.12; $P < 0.05$), compared with vehicle-treated animals (1.46 $\pm$ 0.09); the tubulointerstitial damage index exhibited a similar pattern. The mean glomerular volume was significantly less in estrogen-treated animals. UNX/OVX animals demonstrated significantly greater expression of TGF-β and endothelin-1 in immunohistochemical, *in situ* hybridization, and reverse transcription-PCR assays. This increase was abrogated by estriol but not estradiol. Similarly, significantly higher glomerular and tubulointerstitial expression of proliferating cell nuclear antigen and collagen IV was observed in UNX/OVX animals, and expression was decreased by estriol but not estradiol. It was concluded that, in the UNX model of spontaneous renal damage, glomerular lesions and glomerular hypertrophy were reduced by estriol but less consistently by estradiol. In parallel, loss of podocytes, evidence of podocyte injury (*i.e.*, desmin expression), and expression of mediator systems of glomerular damage were decreased, pointing to a major renoprotective action of estriol.

Several studies have demonstrated that the severity and rate of progression of renal diseases tend to be greater among men, compared with women (1). This is true for several types of renal diseases, such as membranous nephropathy (2), IgA nephropathy (3), and polycystic kidney disease (4). The data from experimental studies are controversial, and very few molecular data are available. Estrogens or estrogen metabolites ameliorate renal damage in some (5) but not all (6,7) models of renal damage. The beneficial effect of estrogen was also noted

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in allograft nephropathy (8). Potential explanations for the discrepancies are that different pathomechanisms operate in the various animal models or that differences in the administered doses, application modalities, and types of estrogen metabolites account for the differences (9–11). In mesangial cell cultures, estrogens (11–13) and selective estrogen receptor modulators (14) reduce proliferation, collagen type IV gene transcription, and collagen protein synthesis.

It has remained unclear whether the presence of testosterone, the absence of estrogen, or both account for the gender differences in renal disease progression (9). In view of the inconsistent results of the experimental studies, we decided to reexamine the effects of estrogens on progression. Because of the magnitude of the gender effect in human disease, we reasoned that the effects of estrogens would not be quantitatively major. We therefore selected the uninephrectomized (UNX), spontaneously hypertensive rat (SHRsp) model, thinking that the relatively small effects might easily go undetected if models of

renal damage with more rapid loss of renal function (*e.g.*, subtotal nephrectomy) were chosen.

There are indications that sex hormones have differential effects in the various regions of the kidney (15,16) and possibly also during compensatory growth after UNX (12). Therefore, an effort was made to quantitate cross-sectional areas of the kidney zones, as well as glomerular volumes, glomerular cell numbers, indices of glomerular injury, and mediators of glomerular injury.

Materials and Methods

Animals

Three-month-old female SHRsp rats were obtained from Prof. U. Ganten (MDC, Berlin, Germany) and were housed in box cages under standard conditions (lights on from 8:00 a.m. to 8:00 p.m., 40 to 70% relative humidity, $22 \pm 1°C$). All animals had free access to water and chow (Altromin 1324, 1% NaCl, soy-free, low in phytoestrogens; Altromin, Lage, Germany).

Animals were randomly allotted to the different experimental groups. For the experimental animals, both ovaries were resected and the right kidney was removed. Animals received 17β-estradiol 3-benzoate (25 μg/d; Aldrich Chemical Co., Milwaukee, WI) dissolved in sesame oil (Sigma Chemical Co., Steinheim, Germany) via subcutaneous injection or received estriol (Incurin; Intervet Pharma, Angers, France; courtesy of Dr. F. Rutten) in the drinking water, with daily control of water consumption and adjustment of the estriol concentration to deliver a daily dose of 0.02 mg/d. The control animals received vehicle.

Experimental Protocol

The rats were randomly allocated to the following five experimental groups: (*1*) sham-operated control animals treated with vehicle (*n* = 9), (*2*) sham-operated control animals treated with 17β-estradiol 3-benzoate (*n* = 10), (*3*) UNX/ovariectomized (OVX) animals treated with vehicle (*n* = 10), (*4*) UNX/OVX animals treated with 17β-estradiol 3-benzoate (*n* = 11), and (*5*) UNX/OVX animals treated with estriol (*n* = 9). Body weights and systolic BP (determined with tail plethysmography) were measured at regular intervals. On two occasions (after 1 mo and before the end of the experiment), the animals were kept in metabolic cages for 1 d, for collection of 24-h urine samples. Urinary albumin excretion was measured with a rat-specific sandwich ELISA system, as described in detail elsewhere (17). The experiment was terminated after 3 mo, with retrograde aortic perfusion.

Tissue Preparation

After perfusion with 3% glutaraldehyde, the remnant kidney was harvested for morphometric investigations. The kidney was sectioned in a plane perpendicular to the interpolar axis, yielding slices of 1-mm width. Ten small pieces of the kidney were selected by area sampling and embedded in Epon-Araldite. Semithin (1 μm) and ultrathin (0.08 μm) sections were prepared and stained with methylene blue/basic fuchsin or lead citrate/uranyl acetate, respectively. The remaining tissue slices were embedded in paraffin; 4-μm sections were stained with hematoxylin/eosin and periodic acid-Schiff stain. To permit immunohistologic investigations, the experiments were repeated and were terminated with perfusion of the kidney with ice-cold NaCl. One-half of the kidney was fixed in 4% buffered formaldehyde, embedded in paraffin, and cut into 2-μm-thick sections. The other half of the kidney was snap-frozen in liquid nitrogen-cooled isopentane for

in situ hybridization. Paraffin sections were prepared and incubated with antibodies, using the avidin-biotin method (17), to detect the following epitopes: desmin (anti-desmin mouse mAb, 1:50; Dako, Carpinteria, CA), proliferating cell nuclear antigen (PCNA) (anti-PCNA mouse mAb, 1:150; Immunotech, Marseille, France), TGF-β_1 (anti-TGF-β_1 rabbit IgG polyclonal antibody, 1:300; Santa Cruz Biotechnology, Santa Cruz, CA), fibronectin (anti-fibronectin rabbit polyclonal antibody, 1:10; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), collagen IV (1:20), estrogen receptor α (anti-mouse estrogen receptor α polyclonal antibody, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and estrogen receptor β (anti-human estrogen receptor β rabbit polyclonal antibody; Alpha Diagnostics, San Antonio, TX). Cryostat sections (5 μm) were prepared and were incubated with antibodies, using the avidin-biotin method, to detect the following antibodies: PDGF-AB (anti-PDGF-AB goat polyclonal antibody, 1:50; Upstate Biotechnology, Waltham, MA), endothelin (ET) (anti-ET-1 rabbit polyclonal antibody, 1:20; Biotrend, Cologne, Germany), and endothelial nitric oxide synthase (eNOS) (anti-eNOS rabbit polyclonal antibody, 1:200; Dianova, Hamburg, Germany).

Estradiol plasma concentrations were measured (Department of Gynecological Endocrinology and Reproductive Medicine, University of Heidelberg, Heidelberg, Germany) with the Elecsys 2010 analytic system (Roche, Mannheim, Germany); the detection threshold was 7 pg/ml. Testosterone plasma concentrations were measured with a RIA (Labor Limbach, Heidelberg, Germany).

Morphologic Investigations

The glomerulosclerosis index (GSI) was assessed on periodic acid Schiff-stained paraffin sections according to the scoring system (scores of 0 to 4) described by el Nahas (18). With the use of light microscopy and a magnification of ×400, the glomerular score for each animal was derived as the arithmetic mean of results for 100 glomeruli (18,19). The tubulointerstitial and vascular damage scores were assessed on periodic acid Schiff-stained paraffin sections at a magnification of ×100, as described previously (20).

The area (A_A) and volume density (V_V) of the renal cortex and medulla, as well as the number of glomeruli per area (N_A), were measured with a Zeiss eyepiece (Integrationsplatte II; Zeiss Co., Oberkochen, Germany) and the point-counting method ($P_P = A_A = V_V$), at a magnification of ×400, as previously described in detail (21). In five semithin sections for each animal, the glomerular cell number and volume were analyzed with a 100-point eyepiece (Integrationsplatte II; Zeiss Co.) for point-counting at a magnification of ×1000 (oil immersion) (21,22). Briefly, glomerular cell numbers (podocytes, mesangial cells, and endothelial cells) were calculated in at least 30 glomeruli for each animal, from cell density per volume (Nc_v) and volume density of the respective cell type (Vc_v), according to the equation $Nc_v = k/\beta \times Nc_A^{1.5}/Vc_v^{0.5}$, with $k = 1$ and $\beta = 1.5$ for podocytes and $\beta = 1.4$ for mesangial and endothelial cells. The respective cell volumes were calculated according to the equation $V_c = Vc_v \times V_{glom}$.

Immunohistologic Investigations

PCNA immunohistochemical reactivity was examined with light microscopy at a magnification of ×400. In 50 glomeruli in each kidney, the numbers of PCNA-positive cells per glomerulus, as well as the numbers per glomerular area and per tubulointerstitial area, were counted. Immunohistologic staining with antibodies against TGF-β_1, ET-1, fibronectin, PDGF, eNOS, estrogen receptors α and β, and collagen I and IV were analyzed by two investigators who were

Table 1. Animal data at the end of the experiment[a]

	Body Weight (g)	Kidney Weight (g)	Systolic BP (mmHg)	Albuminuria (mg/24 h)	17β-Estradiol 3-Benzoate Plasma Concentration (pg/ml)	Testosterone Plasma Concentration (ng/dl)	Uterus Weight (Perfused) (g)
UNX/OVX sham + vehicle (n = 9)	217 ± 9	0.98 ± 0.08[b,c,d]	191 ± 17	1.10 ± 0.46[b]	12.9 ± 9.23[c,e]	16.0 ± 6.6[b,c,d]	1.15 ± 0.2[c]
UNX/OVX sham + 17β-estradiol 3-benzoate (n = 10)	191 ± 7[d]	1. ± 0.06[b,c]	191 ± 24	1.12 ± 0.53[b]	102 ± 60.0	20.5 ± 5.7[b,c,d]	1.32 ± 0.22[c]
UNX/OVX + vehicle (n = 10)	203 ± 9[d]	1.54 ± 0.19[d]	192 ± 23	25.4 ± 8.52	8.0 ± 5.12[c,e]	6.0 ± 2.8	0.49 ± 0.14
UNX/OVX + 17β-estradiol 3-benzoate (n = 11)	192 ± 7[d]	1.63 ± 0.22[d]	209 ± 17	15.4 ± 6.12[b]	101 ± 94.4	9.3 ± 4.13	0.89 ± 0.19[c]
UNX/OVX + estriol (n = 9)	235 ± 13	1.39 ± 0.12	178 ± 21	6.54 ± 2.24[b]	11.3 ± 3.87[c,e]	5.6 ± 2.78	0.74 ± 0.14[c]
ANOVA	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

[a] UNX, uninephrectomized; OVX, ovariectomized.
[b] $P < 0.05$ *versus* UNX/OVX + vehicle.
[c] $P < 0.05$ *versus* UNX/OVX + 17β-estradiol 3-benzoate.
[d] $P < 0.05$ *versus* UNX/OVX + estriol.
[e] $P < 0.05$ *versus* sham + 17β-estradiol 3-benzoate.

blinded with respect to the animal group; scoring was performed as described elsewhere (21).

For analysis of desmin immunohistochemical reactivity, the capillary tuft was divided into four quarters and the following scoring system was used by two investigators who were blinded with respect to the animal group: score 0, no expression; score 1, desmin-positive cells in one quarter; score 2, desmin-positive cells in two quarters; score 3, desmin-positive cells in three quarters; score 4, desmin-positive cells all over the capillary tuft. The damage score was then calculated as $[(0 - \text{score } 0) + \text{score } 1 + \text{score } 2 + \text{score } 3 + \text{score } 4]/5 + 15.$

In Situ Hybridization

Nonradioactive *in situ* hybridization with ET-1, ET_A receptor, and TGF-β sense and antisense probes was performed with five animals in each group, as described elsewhere (21).

Semiquantitative Reverse Transcription-PCR Assays of Renal ET-1, ET_A and ET_B Receptor, and TGF-β mRNA Expression

Semiquantitative reverse transcription-PCR for renal ET-1, ET_A and ET_B receptor, and TGF-β mRNA expression was performed with five animals in each group, as described elsewhere (21).

Statistical Analyses

Data are given as mean ± SD. After testing for normal distribution, the Kruskal-Wallis test or one-way ANOVA, followed by Duncan's multiple-range test, was chosen to test for differences between groups. The results were considered significant when the *P* value was <0.05.

Results

Animal Data

In UNX/OVX animals, administration of estradiol, but not estriol, was associated with decreased food consumption (Tables 1 and 2). There were no significant differences in body weights between the groups, with the exception of higher body weights for estriol-treated UNX/OVX animals. The kidney weight/body weight ratio was significantly higher for UNX/OVX animals. The weights of the perfusion-fixed uteri were significantly lower in vehicle-treated UNX/OVX animals, compared with the other experimental groups.

There were no significant differences in BP between the groups. In UNX/OVX animals, a striking increase in albuminuria was observed. Albumin excretion was significantly decreased by both estrogens. Estradiol plasma concentrations were higher in estradiol-treated sham-operated and UNX/OVX animals. No change in estradiol plasma concentrations was observed in estriol-treated animals. Testosterone plasma concentrations were lower in all UNX/OVX groups, compared with sham-operated animals, and were not affected by estrogen treatment.

Creatinine and urea concentrations were significantly higher in vehicle-treated UNX/OVX animals, compared with sham-operated animals. Estrogen treatment significantly decreased creatinine concentrations, but only estriol decreased the urea concentration as well (Table 2).

Table 2. Plasma concentrations of selected compounds at the end of the experiment

	Creatinine (mg/dl)	Urea (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL Cholesterol (mg/dl)	LDL Cholesterol (mg/dl)
UNX/OVX sham + vehicle (n = 9)	0.55 ± 0.18[a]	53.0 ± 17.4[a,b]	107 ± 14.6[a,b,c]	69.1 ± 17.04[b]	64.3 ± 5.96[a,c]	29.22 ± 12.78[a,b]
UNX/OVX sham + 17β-estradiol 3-benzoate (n = 10)	0.48 ± 0.06[a,b]	44.8 ± 5.07[a,b]	107 ± 16.3[a,b,c]	83.9 ± 25.3[b]	60.7 ± 8.31[a,b,c]	29.1 ± 12.64[a,b]
UNX/OVX + vehicle (n = 10)	0.68 ± 0.15	71.5 ± 11.1	148 ± 24.0	73.8 ± 32.4[b]	87.6 ± 11.7	45.2 ± 11.9
UNX/OVX + 17β-estradiol 3-benzoate (n = 11)	0.63 ± 0.09[a]	72.8 ± 16.03	144 ± 31.3	117 ± 30.2	72.2 ± 18.3	47.56 ± 18.3
UNX/OVX + estriol (n = 9)	0.53 ± 0.06[a]	55.4 ± 4.6[a,b]	133 ± 16.3	75.0 ± 21.3[b]	83.1 ± 6.62	35.0 ± 14.7
ANOVA	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

[a] $P < 0.05$ *versus* UNX/OVX + vehicle.
[b] $P < 0.05$ *versus* UNX/OVX + 17β-estradiol 3-benzoate.
[c] $P < 0.05$ *versus* UNX/OVX + estriol.

Morphologic Findings

UNX/OVX animals demonstrated a significantly higher GSI, compared with sham-operated rats (Table 3). The GSI was significantly lower in estradiol- and estriol-treated rats, compared with vehicle-treated rats. The tubulointerstitial damage index was significantly higher in UNX/OVX rats, compared with control animals. The tubulointerstitial damage index was lower in estriol-treated but not estradiol-treated animals. The vascular damage index was also significantly higher in UNX/OVX animals, compared with control animals. Both interventions significantly decreased the vascular damage index in UNX/OVX animals.

The mean glomerular volume was significantly higher in vehicle-treated UNX/OVX animals, compared with sham-operated animals. Both estrogens decreased the mean glomerular volume in UNX/OVX animals.

The number of podocytes was significantly lower in vehicle-treated UNX/OVX rats than in vehicle-treated sham-operated animals (Table 4). The mean podocyte volume was not modified by any intervention. The mesangial cell number was higher in vehicle-treated UNX/OVX rats, compared with vehicle-treated sham-operated control animals. The mesangial cell number was lower in estriol-treated but not estradiol-treated animals. The mesangial cell volume was also higher in vehicle-treated UNX/OVX rats, compared with sham-operated control rats and treated UNX/OVX rats. Finally, the endothelial cell number per glomerulus was significantly higher in all UNX/OVX groups, irrespective of treatment. The mean endothelial cell volume was also higher in UNX/OVX rats, compared with sham-operated control rats. Both interventions significantly decreased the endothelial cell volume.

Capillary length density and total glomerular capillary length were significantly lower in estradiol-treated animals than in estriol-treated UNX/OVX animals (Table 5). The fractional glomerular capillary tuft volume demonstrated no difference between the groups. The fractional mesangial matrix volume was highest in vehicle-treated UNX/OVX animals and was lower in estriol-treated but not estradiol-treated animals.

Immunohistochemical Findings

Podocyte staining for desmin, as an early marker of podocyte damage, was demonstrable in untreated UNX/OVX animals (0.48 ± 0.31), compared with vehicle-treated sham-operated control animals (0.04 ± 0.02) (Figure 1, A to D). Estriol treatment (0.05 ± 0.01) but not estradiol treatment (0.68 ± 0.32) significantly reduced desmin staining in podocytes ($P < 0.05$). The number of PCNA-positive cells, as an index of glomerular cell proliferation, was significantly increased in UNX/OVX animals; values were not significantly affected by estradiol but were significantly decreased by estriol treatment (Figure 2).

Glomerular expression and, more markedly, tubulointerstitial expression of ET-1 protein were significantly greater in UNX/OVX animals. Expression was not significantly altered in estradiol-treated animals and was slightly but significantly lower in estriol-treated animals. As demonstrated in Figure 2B, a similar relationship was observed for eNOS expression.

Table 3. Renal damage indices and glomerular geometric parameters[a]

	GSI	TBI	VI	Glomerular Number/Kidney	Mean Glomerular Volume ($\times 10^6$ μm^3)	Cortex Volume (cm^3)	Medulla Volume (cm^3)
UNX/OVX sham + vehicle (n = 9)	0.38 ± 0.08[b,c]	0.68 ± 0.09[b,c]	0.53 ± 0.08[b]	42,272 ± 3973	1.08 ± 0.09[b]	0.61 ± 0.04[b,c,d]	0.33 ± 0.03[b,c]
UNX/OVX sham + 17β-estradiol 3-benzoate (n = 10)	0.34 ± 0.13[b,c]	0.47 ± 0.16[b,c]	0.48 ± 0.12[b]	40,333 ± 1973	1.25 ± 0.15[b]	0.65 ± 0.03[b,c,d]	0.32 ± 0.03[b,c]
UNX/OVX + vehicle (n = 10)	1.46 ± 0.09	1.08 ± 0.16	0.78 ± 0.17	38,031 ± 2735	1.64 ± 0.2[c,d]	0.87 ± 0.07	0.47 ± 0.04[c,d]
UNX/OVX + 17β-estradiol 3-benzoate (n = 11)	0.69 ± 0.16[b]	0.95 ± 0.1[b]	0.51 ± 0.11[b]	38,623 ± 4450	1.12 ± 0.16	0.84 ± 0.12	0.43 ± 0.03
UNX/OVX + estriol (n = 9)	0.21 ± 0.12[b,c]	0.60 ± 0.26[b,c]	0.48 ± 0.11[b]	38,623 ± 9192	1.12 ± 0.38	0.84 ± 0.09	0.41 ± 0.01
ANOVA	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$

[a] GSI, glomerulosclerosis index; TBI, tubulointerstitial damage index; VI, vascular damage index.
[b] $P < 0.05$ *versus* UNX/OVX + vehicle.
[c] $P < 0.05$ *versus* UNX/OVX + 17β-estradiol 3-benzoate.
[d] $P < 0.05$ *versus* UNX/OVX + estriol.

The protein expression of TGF-β was significantly ($P < 0.05$) higher in the glomeruli (0.72 ± 0.06) and interstitial spaces (1.86 ± 0.21) of UNX/OVX animals, compared with glomerular (0.11 ± 0.13) and tubulointerstitial (0.3 ± 0.23) expression in vehicle-treated sham-operated animals (Figure 1, E to H). Expression was not significantly changed in estradiol-treated animals (glomerular, 0.52 ± 0.08; tubulointerstitial, 1.98 ± 0.83) but was definitely decreased in estriol-treated animals (glomerular, 0.1 ± 0.06; tubulointerstitial, 0.84 ± 0.55).

Collagen IV staining was significantly ($P < 0.05$) upregulated in UNX/OVX animals (glomerular, 3.1 ± 0.42; tubulointerstitial, 2.7 ± 0.26), compared with vehicle-treated sham-operated animals (glomerular, 1 ± 0.25; tubulointerstitial, 1.58 ± 0.33). Staining was little changed with estradiol (glomerular, 2.6 ± 0.4; tubulointerstitial, 2.66 ± 0.2), but glomerular expression was significantly less in estriol-treated animals (glomerular, 2.4 ± 0.68; tubulointerstitial, 2.53 ± 0.53).

Glomerular expression of estrogen receptor α was significantly increased in UNX /OVX animals (2.63 ± 0.39), compared with vehicle-treated sham-operated control animals (0.63 ± 0.49). Estradiol had no effect on glomerular expression (2.32 ± 0.26) but estriol reduced staining (1.03 ± 0.06, $P < 0.05$), compared with UNX /OVX animals. Glomerular expression of estrogen receptor β was significantly elevated in vehicle-treated UNX /OVX rats (2.04 ± 0.35), compared with vehicle-treated sham-operated animals (1.01 ± 0.67). Both estradiol (0.89 ± 0.96) and estriol (1.21 ± 0.17) reduced expression.

ET-1, ET$_A$ Receptor, and TGF-β mRNA Expression Determined by Nonradioactive In Situ Hybridization

Glomerular expression and, more markedly, tubulointerstitial expression of ET-1 mRNA were significantly higher in vehicle-treated UNX/OVX animals (glomerular, 1.35 ± 0.07; tubulointerstitial, 1.6 ± 0.16), compared with vehicle-treated sham-operated animals (glomerular, 0.87 ± 0.51; tubulointerstitial, 0.98 ± 0.42). Expression was not significantly altered in estradiol-treated animals (glomerular, 1.46 ± 0.37; tubulointerstitial, 1.61 ± 0.3) but was significantly lower in estriol-treated animals (glomerular, 0.72 ± 0.32; tubulointerstitial, 0.67 ± 0.38).

The expression of ET$_A$ receptor mRNA changed similarly in the glomeruli and the tubulointerstitium. Expression was higher in vehicle-treated UNX/OVX animals (glomerular, 1.19 ± 0.15; tubulointerstitial, 1.54 ± 0.09; $P < 0.05$) and estradiol-treated UNX/OVX animals (glomerular, 1.53 ± 0.17; tubulointerstitial, 1.87 ± 0.27), compared with vehicle-treated sham-operated animals (glomerular, 0.51 ± 0.35; tubulointerstitial, 0.67 ± 0.31) and estriol-treated UNX/OVX animals (glomerular, 0.49 ± 0.29; tubulointerstitial, 0.52 ± 0.2; $P < 0.05$).

The expression of TGF-β mRNA was significantly higher ($P < 0.05$) in the glomeruli (1.96 ± 0.19) and the interstitial spaces (1.43 ± 0.21) in vehicle-treated UNX/OVX animals, compared with glomerular (0.61 ± 0.31) and tubulointerstitial (0.69 ± 0.42) expression in vehicle-treated sham-operated

Table 4. Glomerular cells

	Podocyte Number/ Glomerulus	Mean Podocyte Volume (μm^3)	Mesangial Cell Number/ Glomerulus	Mean Mesangial Cell Volume (μm^3)	Endothelial Cell Number/ Glomerulus	Mean Endothelial Cell Volume (μm^3)
UNX/OVX sham + vehicle ($n = 9$)	110 ± 15[a]	475 ± 56	112 ± 41[a,b]	173 ± 43[a]	116 ± 27[a,b,c]	144 ± 45[a]
UNX/OVX sham + 17β-estradiol 3-benzoate ($n = 10$)	97 ± 21	361 ± 126	143 ± 33[a,b]	184 ± 52[a]	124 ± 38[a,b]	129 ± 23[a]
UNX/OVX + vehicle ($n = 10$)	67 ± 12	478 ± 116	206 ± 35	303 ± 142[b,c]	166 ± 40	218 ± 31[b,c]
UNX/OVX + 17β-estradiol 3-benzoate ($n = 11$)	96 ± 38	468 ± 226	263 ± 57	198 ± 64	186 ± 24	121 ± 43
UNX/OVX + estriol ($n = 9$)	84 ± 35	390 ± 137	167 ± 45[a,b]	127 ± 143	175 ± 42	110 ± 21
ANOVA	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

[a] $P < 0.05$ *versus* UNX/OVX + vehicle.
[b] $P < 0.05$ *versus* UNX/OVX + 17β-estradiol 3-benzoate.
[c] $P < 0.05$ *versus* UNX/OVX + estriol.

Table 5. Length density of glomerular capillaries (L_V), total length of glomerular capillaries (L_{total}), fractional glomerular capillary tuft volume, and fractional mesangial matrix volume

	L_V (mm/mm^3)	L_{total} (m/Kidney)	Fractional Glomerular Capillary Tuft Volume (% of Glomerulus)	Fractional Mesangial Matrix Volume (% of Tuft)
UNX/OVX sham + vehicle ($n = 9$)	11,330 ± 2705[a]	1250 ± 364[a]	79.7 ± 3.19	1.94 ± 0.33[a,b]
UNX/OVX sham + 17β-estradiol 3-benzoate ($n = 10$)	9421 ± 2584[a]	1198 ± 371[a]	80.3 ± 3.28	2.00 ± 0.49[a,b]
UNX/OVX + vehicle ($n = 10$)	8554 ± 953[a]	1334 ± 181[a]	74.5 ± 4.51	5.43 ± 0.97
UNX/OVX + 17β-estradiol 3-benzoate ($n = 11$)	8225 ± 2106	928 ± 309	76.0 ± 4.15	5.25 ± 0.66
UNX/OVX + estriol ($n = 9$)	9779 ± 2368[a]	1260 ± 595[a]	78.5 ± 4.20	2.88 ± 0.49[a,b]
ANOVA	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$

[a] $P < 0.05$ *versus* UNX/OVX + 17β-estradiol 3-benzoate.
[b] $P < 0.05$ *versus* UNX/OVX + vehicle.

animals. Expression was not significantly changed in estradiol-treated animals (glomerular, 1.35 ± 0.1; tubulointerstitial, 1.76 ± 0.14) but was definitely less in estriol-treated animals (glomerular, 0.32 ± 0.39; tubulointerstitial, 0.44 ± 0.59).

Reverse Transcription-PCR Assays for TGF-β, ET-1, and ET$_A$ and ET$_B$ Receptors

The TGF-β mRNA ratio was higher in vehicle-treated UNX/OVX animals (1.03 ± 0.69) and estradiol-treated UNX/OVX animals (1.22 ± 0.35; $P < 0.05$), compared with vehicle-treated sham-operated animals (0.57 ± 0.38). The ratio was significantly decreased after estriol treatment (0.58 ± 0.24). The ET-1 mRNA ratio was significantly elevated in all UNX/OVX animals, compared with vehicle-treated sham-operated

animals. None of the treatments had a significant effect on ET-1 expression.

The ET$_A$ and ET$_B$ receptor mRNA ratios were significantly higher in vehicle-treated UNX/OVX animals (ET$_A$, 1.16 ± 0.43; ET$_B$, 2.06 ± 0.63) and estradiol-treated UNX/OVX animals (ET$_A$, 1.23 ± 0.47; ET$_B$, 2.42 ± 0.64), compared with sham-operated control animals (ET$_A$, 0.66 ± 0.16; ET$_B$, 1.64 ± 0.61). Estriol significantly reduced the increase in expression of both receptors (ET$_A$, 0.69 ± 0.23; ET$_B$, 1.39 ± 0.55).

Discussion

The main finding of this study is the demonstration that estriol (and to a lesser extent 17β-estradiol 3-benzoate) decreases albuminuria, as well as indices of glomerular and
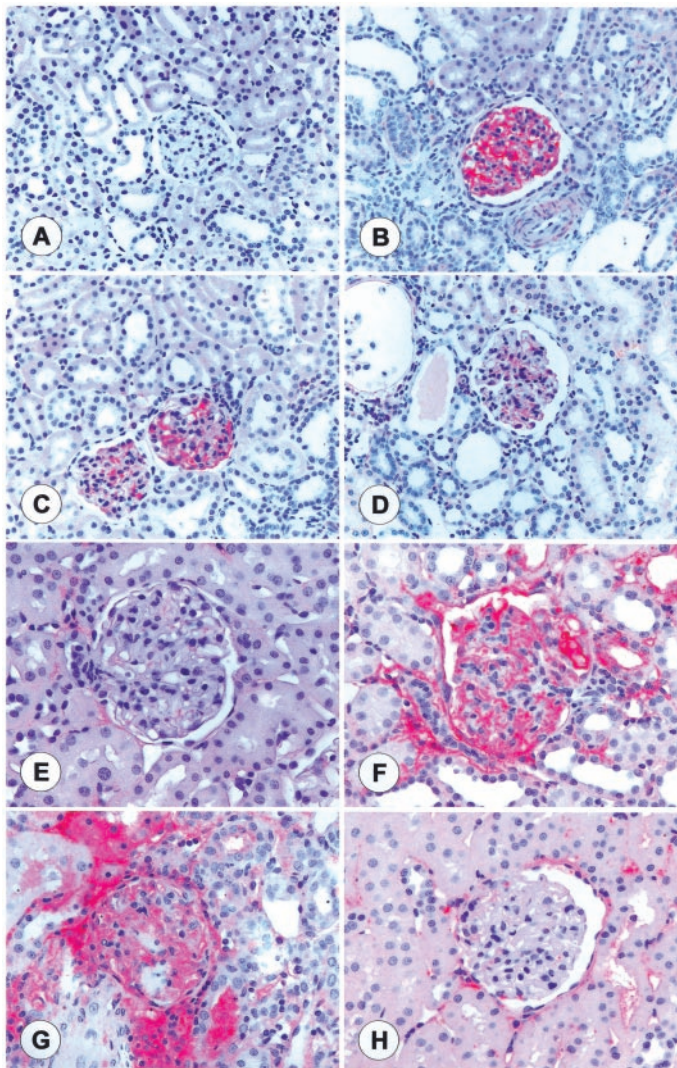
Figure 1. Effects of 17β-estradiol 3-benzoate and estriol on the expression of desmin (A to D) (immunohistochemical staining; magnification, ×250) and TGF-β (E to H) (immunohistochemical staining; magnification, ×300). (A) Vehicle-treated sham-operated animal, with virtually no desmin expression. (B) Vehicle-treated uninephrectomized (UNX)/ovariectomized (OVX) rat, with marked glomerular desmin expression by podocytes. (C) 17β-Estradiol 3-benzoate-treated UNX/OVX rat, with some desmin expression. (D) Estriol-treated UNX/OVX rat, with low desmin expression. (E) Vehicle-treated sham-operated animal, with low baseline TGF-β expression. (F) Vehicle-treated UNX/OVX rat, with marked TGF-β expression. (G) 17β-Estradiol 3-benzoate-treated UNX/OVX rat, with less pronounced TGF-β expression. (H) Estriol-treated UNX/OVX rat, with borderline TGF-β expression.

tubulointerstitial damage and expression of systems mediating renal injury, in a model of spontaneous progressive renal damage (*i.e.*, UNX SHRsp rats). Treatment with estriol affected the volume and cellular composition of the glomeruli, prevented podocyte injury, and attenuated upregulation of ET-1 and its receptors, TGF-β, and estrogen receptor α at the mRNA and protein levels. Because the estrogens were administered from the beginning of the study, the study investigated

prevention of injury and not regression of established renal injury.

The estrogens in this study were administered continuously, as in comparable studies (5). We admit that cycling concentrations may have effects different from those noted in our study.

The beneficial effects of estrogens in this model cannot be explained on the basis of simple artifacts, such as effects on lipid levels, body weights, or BP. LDL and total cholesterol levels were increased in UNX/OVX animals but were not affected by estrogen treatment. Despite similar food consumption, body weights were higher for estriol-treated UNX/OVX animals, compared with vehicle-treated UNX/OVX animals. Therefore, the beneficial effect of estriol certainly cannot be explained on the basis of malnutrition and wasting. There was also no significant difference in systolic BP, although we note that values were lower for estriol-treated animals.

In UNX/OVX animals, the weight of the remnant kidney increased. This increase was associated with a marked increase in the mean glomerular volume and cortical volume. Estriol and estradiol treatment reduced the glomerular volume but not the cortical volume, indicating dissociation between glomerular and tubular growth.

The GSI was higher in vehicle-treated UNX/OVX rats; it was normalized by estriol and significantly reduced by estradiol. Similar patterns were observed for indices of tubulointerstitial and vascular damage. Compared with sham-operated animals, the mean podocyte number was significantly lower in UNX/OVX animals, presumably reflecting podocyte loss. There was a trend toward higher podocyte numbers in estriol- and estradiol-treated animals. Definitive evidence ($P < 0.05$) of podocyte damage was provided by the expression of desmin, a sensitive marker of early podocyte injury (23), and expression was virtually abrogated by estriol treatment.

Striking increases in the mesangial cell number and volume were noted; these were abrogated by estriol and, to a lesser extent, estradiol. The changes in the mesangial cell volume and the increase in the mesangial matrix proceeded in parallel. The mesangial cell number was significantly lower in estriol-treated animals, and the mesangial cell volume was decreased by both estriol and estradiol treatments.

Finally, the endothelial cell number and volume were significantly increased in UNX/OVX animals, presumably reflecting intensive capillary remodeling. In parallel, the number of PCNA-positive cells increased. The endothelial cell volume was normalized by estradiol and estriol treatments. This finding is not implausible, because at least arterial endothelial cells express estrogen receptors (24).

The evidence of a renoprotective action of estrogens, particularly estriol, on structural parameters was complemented by evidence of less pronounced expression of mediator systems known to operate in the pathogenesis of glomerular and tubulointerstitial injury. This was particularly true for TGF-β and ET-1, at both the protein and mRNA levels. As anticipated from the changes in glomerular and tubulointerstitial damage indices, the expression of collagen IV was increased in UNX/

OVX animals and this was partially abrogated by estriol treatment.

For the interpretation of the findings of less glomerular, interstitial, and vascular fibrosis, it is of interest that the effects of estrogens on fibroblasts differ in various organs. 17β-Estradiol 3-benzoate was demonstrated to be pro-proliferative in connective tissue (25) and in skin and lung fibroblasts (26) but to diminish cell proliferation in the renal ablation model (5). Estrogens normalized wound bed collagen contents and structure in the OVX rat model (27). In mesangial cell cultures, genistein, a selective estrogen receptor modulator (14), and 17β-estradiol 3-benzoate reduced expression of collagen types I and IV (28). 17β-Estradiol 3-benzoate also upregulated expression of metalloproteinases (28,29). In view of the potential role of hypoxia in the promotion of renal fibrosis (30), it is of interest that 17β-estradiol 3-benzoate was demonstrated to induce the expression of hypoxia-inducible genes, such as vascular endothelial growth factor (31), but suppressed ET-1 production in the kidney (32). In our study, estriol but not estradiol reduced expression of ET-1 and ET receptors.

Estrogens also stimulate the renin-angiotensin system and increase angiotensinogen concentrations but reduce plasma renin activity in the adrenal gland and possibly also in the kidney (33). Estrogens further decrease AT_1 receptor expression (34), possibly by modulating the adrenal concentrations of angiotensin II (AngII) (34), and increase the expression of AT_2 receptors (35). This might be relevant to explain the findings of less glomerular growth and less fibrosis in our study. In vascular smooth muscle cells, estrogens antagonize the growth-promoting effects of AngII (32). Estrogens inhibit the AngII-induced stimulation of NAD(P) oxidase in endothelial cells (36). Not only the response to AngII but also that to α-adrenoreceptors is attenuated by estrogens (37).

Estriol was more effective in abrogating renal injury. This difference between estriol and estradiol remains intriguing. We

Figure 2. Effects of 17β-estradiol 3-benzoate and estriol on the expression of endothelin-1 (ET-1), proliferating cell nuclear antigen (PCNA), and endothelial nitric oxide synthase (eNOS), as assessed immunohistochemically. □, Glomerular expression; ■, tubulointerstitial expression. (A) Frequency of PCNA-positive cells (per glomerular section and per tubulointerstitial field of view, respectively). A consistent increase in PCNA-positive cells in the glomeruli and the tubulointerstitial spaces was observed in UNX/OVX animals. 17β-Estradiol 3-benzoate and more markedly estriol reduced the number of PCNA-positive cells/glomerulus. Estriol but not 17β-estradiol 3-benzoate reduced the number of PCNA-positive cells in the tubulointerstitial space. (B) Tubulointerstitial expression of ET-1. Expression was elevated in vehicle-treated UNX/OVX rats and was even higher in 17β-estradiol 3-benzoate-treated rats. Estriol significantly decreased expression. (C) Glomerular and tubulointerstitial expression of eNOS. Expression was elevated in UNX/OVX animals and remained high in 17β-estradiol 3-benzoate-treated animals. Estriol decreased glomerular and tubulointerstitial expression of eNOS. ●, $P < 0.05$ *versus* operation (op) plus vehicle; ◆, $P < 0.05$ *versus* operation plus estradiol; ◈, $P < 0.05$ *versus* operation plus estriol; ■, $P < 0.05$ *versus* sham operation plus estradiol; ★, $P < 0.05$ *versus* sham operation plus vehicle.
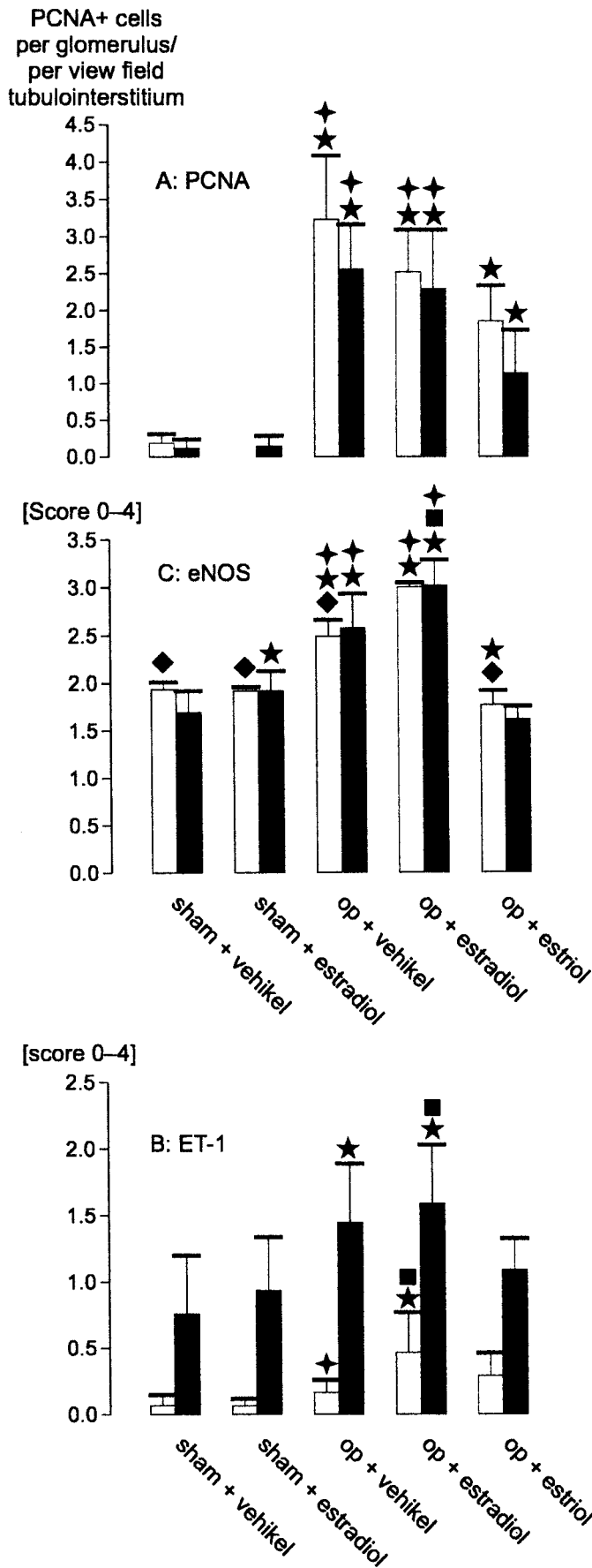
do not have information on the dose-response relationship, but we can exclude the possibility of insufficient estradiol absorption from the subcutaneous depot, because increased estradiol plasma concentrations were measured. We acknowledge the difference in the concentration-time profiles (with drinking water administration for estriol *versus* continuous release from the subcutaneous oil depot for estradiol). We also note that the effects of oral, compared with parenteral, administration may differ because of metabolism of estrogens in the intestinal wall and the liver (with a number of secondary consequences, such as alterations in angiotensinogen concentrations and sex hormone-binding protein levels). We also cannot exclude the possibility of differences in effector pathways, particularly with respect to the relative contributions of genomic *versus* nongenomic pathways. There is recent evidence that estriol also acts via nongenomic pathways. There are two known estrogen receptor types (estrogen receptors α and β), and recent data suggest that the protective effects of estrogens are mediated mainly by estrogen receptor α (38). This is of interest, because glomerulosclerosis-prone and -resistant mice differ in expression, with decreased expression of receptors α and β in the glomeruli of glomerulosclerosis-prone mice (29). 17β-Estradiol decreased collagen accumulation and increased matrix metalloproteinase-9 expression and activity (39). The action of the estrogen receptor is markedly influenced by specific coregulators that enhance transcriptional activity by recruiting the repressor of estrogen activity away from the estrogen receptor (40). It has been proposed that the antiproliferative actions of 17β-estradiol 3-benzoate are mediated by local conversion to hydroxy and methoxy metabolites, which exhibit specific effects despite having no interaction with estrogen receptor α (41). The renal metabolism of estrogens also displays some unique aspects that may be particularly relevant for the pathogenesis of estrogen-induced renal tumors (42).

In view of the heterogeneous effects of estrogens in renal pathologic conditions (5–7), it is noteworthy that some authors observed cell type-dependent differences in estrogen-induced mitogen-activated protein kinase pathways (43). Estrogens also influence factors that may be involved in renal pathologic conditions, such as insulin-like growth factor (44), heat shock proteins (45), and expression of antioxidant (46) or 11β-hydroxysteroid dehydrogenase (47) enzymes. In view of the strikingly renoprotective effects of retinoids (48), it is also of interest that estrogens cause coordinate upregulation of retinoid production and retinoid signaling and have antiproliferative actions as well (49). In this *in vivo* model of spontaneous mild renal damage, administration of estrogens attenuated the development of renal injury. The gender-dependent difference in renal damage is not completely explained by the absence of testosterone, consistent with the idea of a specific renoprotective action of estrogens (29). There are marked species differences with respect to the effects of estrogens, and it is therefore not possible to extrapolate these data directly to human subjects. The discovery of selective estrogen modulators without feminizing actions might provide an additional stimulus for addressing this issue in human subjects.

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