2-Hydroxyestradiol Attenuates Renal Disease in Chronic Puromycin Aminonucleoside Nephropathy

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Abstract. It has been previously shown that 2-hydroxyestradiol (2-OHE) attenuates the development of renal disease in genetic nephropathy associated with obesity and the metabolic syndrome. The purpose of this study was to test the hypothesis that 2-OHE, irrespective of its effects on metabolic status and/or obesity, exerts direct renoprotective effects *in vivo*. First, the effects of increasing doses of 2-OHE on mesangial cell growth, proliferation, and collagen synthesis in isolated rat glomerular mesangial cells were evaluated *in vitro*. Second, the effects of 12-wk administration of 2-OHE (10 μg/h per kg) on renal function and structure in chronic puromycin aminonucleoside (PAN)–induced nephropathy in rats were evaluated *in vivo*. 2-OHE concentration-dependently (0.001 to 1 μmol/L; $P < 0.001$) inhibited serum (2.5%)–induced cell growth (^{3}H-thymidine incorporation), collagen synthesis (^{3}H-proline incorporation), and cell proliferation (cell number). Importantly, the inhibitory effects of 2-OHE (0.1 μmol/L) were not blocked by ICI182780 (50 μmol/L), an estrogen receptor antagonist. *In vivo*, chronic administration of PAN (75 mg/kg + 5 × 20 mg/kg) over 12 wk induced severe chronic renal disease.

Chronic treatment with 2-OHE significantly ($P < 0.05$) attenuated PAN-induced decrease in glomerular filtration, reduced proteinuria, and the elevated BP, and it had no effect on PAN-induced increase in plasma cholesterol and triglycerides levels. 2-OHE had no effects on plasma testosterone levels in male nephropathic animals. Immunohistochemical staining for collagen IV and proliferating cell nuclear antigen (PCNA) in glomeruli and transforming growth factor–β (TGF-β) in renal tubular cells were significantly higher in PAN nephropatic rats *versus* control animals with intact kidneys. PAN also markedly increased glomerular and interstitial macrophage infiltration (ED1$^+$ cells). 2-OHE had no effects on renal tubular cell TGF-β, but it significantly reduced glomerular PCNA and collagen IV and glomerular and interstitial macrophage infiltration. In summary, this study provides the first evidence that 2-OHE exerts direct renoprotective effects *in vivo*. These effects are mediated by estrogen receptor-independent mechanisms and are due, at least in part, to the inhibition of some of the key proliferative mechanisms involved in glomerular remodeling and sclerosis.

Recent data indicate that the rate of progression of chronic renal disease (CRD) of various etiologies is more rapid in men than in women and is independent of BP or serum cholesterol levels (1,2). Accelerated arteriosclerosis and excessive rate of cardiovascular morbidity and mortality characterize CRD (3), and, similarly to cardiovascular disease, the incidence and prevalence of CRD is higher in men than in women (4). Although the involvement of genetic factors, environment, and androgens should not be neglected, the resistance of kidneys in women to the progression of renal disease is most frequently attributed to estrogens (2).

Our recent studies suggest that several of the cellular effects of 17β-estradiol are mediated by its non-estrogenic metabolites, particularly the catecholestradiols (2-hydroxyestradiol and 4-hydroxyestradiol). For example, the inhibitory effects 17β-estradiol on vascular smooth muscle cell and cardiac fibroblast proliferation and collagen production and endothelin-1 production by endothelial cells are mediated, at least in part, by the metabolism of 17β-estradiol to 2-hydroxyestradiol (5–8).

The oxidative metabolism of 17β-estradiol determines the nature of the biologic effects of this endogenous estrogen. In this regard, C16 hydroxylation leads to the production of estriol, a very active estrogen with high uterotropism (9). In contrast, C2 hydroxylation leads to the formation of 2-hydroxyestradiol (2-OHE), a metabolite that has no uterotropic activity (10), has very low affinity for estrogen receptors (11), and is cleared from plasma 10 times faster than 17β-estradiol (12). It is conceivable, therefore, that 2-OHE may be effective and safe to use in both men and women.

We recently examined the potential cardiovascular and renal protective effects of 2-OHE in male obese ZSF1 rats, a genetic model of obesity and the metabolic syndrome (*i.e.*, hypertension, insulin resistance, and hyperlipidemia) that develops nephropathy characterized by massive proteinuria, reduced GFR, and abnormal renal histopathology (13,14). 2-Hydroxyestra-
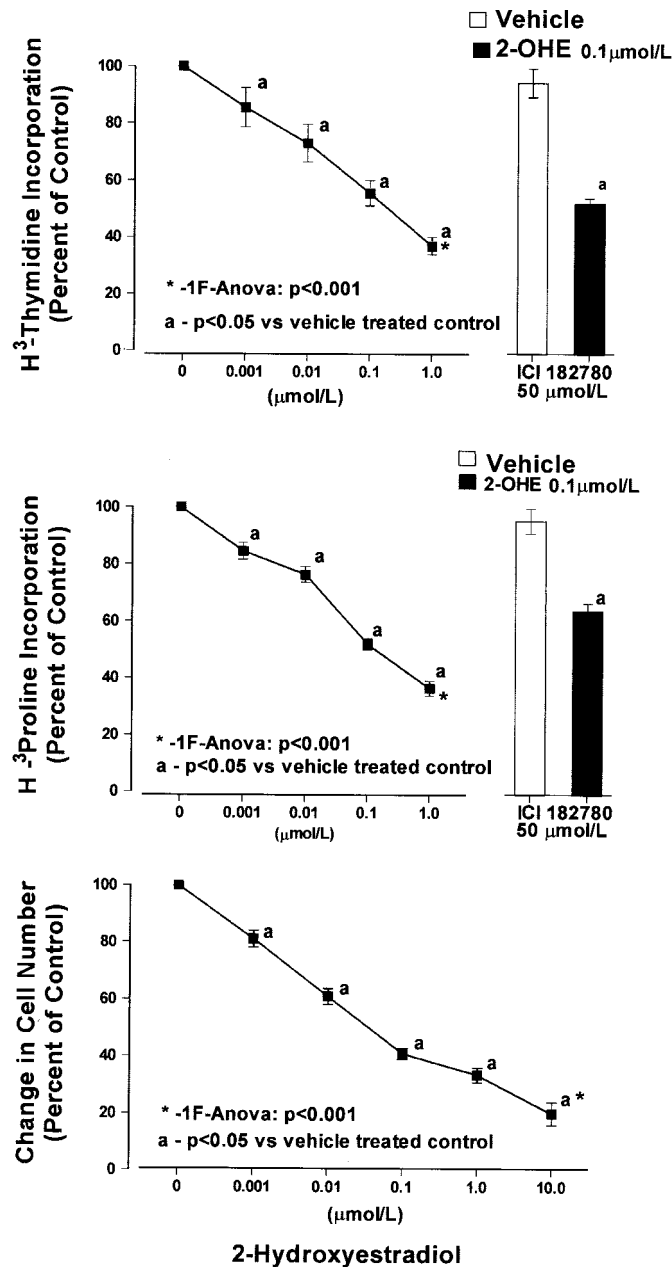
Figure 1. The inhibitory effects of 2-hydroxyestradiol on fetal calf serum (FCS)–induced growth (^{3}H-thymidine incorporation, top panel), collagen synthesis (^{3}H-proline incorporation, middle panel), and cell proliferation (bottom panel) of rat mesangial cells.

diol attenuated the development of obesity and improved endothelial function, decreased the severity of diabetes, lowered arterial BP, and reduced plasma cholesterol and proteinuria (15). 2-OHE also reduced the extent of tubulointerstitial damage and glomerulosclerosis (16), suggesting that this estradiol metabolite may provide significant renoprotection in diseases with high risk for renal injury (*i.e.*, obesity and the metabolic syndrome). Although our previous *in vitro* studies suggest that 2-OHE may have direct renoprotective effects, the observed renoprotective effects in obese ZSF1 rats might be due to the reduced food intake and obesity and improved glucose control

and lipid status. Therefore, the purpose of this study was to test the hypothesis that 2-OHE exerts direct renoprotective effects *in vivo*. We used the chronic puromycin-aminonucleoside (PAN) model in which the repeated injections of low doses of PAN induce nephropathy that resembles focal segmental glomerulosclerosis (FSGS; [17]). In addition, we studied the effects of 2-OHE on rat mesangial cells growth, proliferation, and collagen synthesis. This study provides the first evidence that 2-OHE, an estradiol metabolite with little estrogen activity, has direct renoprotective effects in a rodent model of chronic renal failure with FSGS.

Materials and Methods
In Vitro Growth Studies in Rat Mesangial Cells

Mesangial Cell Culture. Glomerular mesangial cells (GMC) were grown as explants from glomeruli isolated from male, 12- to 15-wk-old Sprague Dawley rats and as described previously (18,19). GMC were grown under standard tissue culture conditions in phenol red free DMEM/F12 supplemented with 10% charcoal-stripped fetal calf serum (19). Confluent GMC were dislodged by trypsinization, washed, and plated for growth studies at required densities in multi-well plates. GMC in second or third passage were used for growth (DNA synthesis, collagen synthesis, and cell proliferation) studies.

DNA and Collagen Synthesis. ^{3}H-Thymidine and ^{3}H-proline incorporation studies were performed as measures of DNA and collagen synthesis, respectively. GMC were plated at a density of 2.5×10^4 cells/well in 24-well tissue culture dishes and allowed to grow in DMEM/F12 containing 10% fetal calf serum (FCS) under standard tissue culture conditions. The monolayers of GMC were then growth arrested by feeding DMEM containing 0.4% bovine serum albumin (BSA) for 48 h. Growth was stimulated by treating growth arrested GMC with DMEM supplemented with 2.5% FCS and containing or lacking the various treatments. For DNA synthesis, after 20 h of incubation, the cells were pulsed with ^{3}H-thymidine (1μCi/ml) for an additional 4 h. For collagen synthesis, the cells were treated for 48 h in the presence of ^{3}H-L-proline (1μCi/ml). The experiments were terminated by washing the cells twice with Dulbecco's phosphate-buffered saline (PBS) and twice with ice-cold TCA (10%). The precipitate was dissolved in 0.5 ml of 0.3 N NaOH and 0.1% sodium dodecyl sulfate (SDS) after incubation at 50°C for 2 h. Aliquots from four wells for each treatment with 10 ml scintillation fluid were counted in a liquid scintillation counter, and each experiment was conducted using three to four separate cultures. ^{3}H-Thymidine incorporation studies were conducted in sub-confluent monolayers. However, to ensure that changes in collagen synthesis were not due to decreases in cell number, ^{3}H-proline incorporation studies were conducted in confluent monolayers of cells in which changes in cell number were precluded (20).

Cell Proliferation. Cell counting was performed as a direct measure of cell proliferation. Trypsinized GMC were suspended in DMEM/F12 containing 10% FCS and plated in a 24-well culture dish at a density of 1×10^4 cells/well. After incubation for 24 h, cells were growth arrested by feeding DMEM containing 0.4% BSA for 48 h. GMC were then treated every 24 h for 4 d with DMEM supplemented with 2.5% FCS and containing or lacking various treatments. The treatments were terminated on day 5, and cells were dislodged with trypsin-EDTA, diluted in Isoton-II, and counted with a Coulter counter. Aliquots from three wells were counted for each group and using three separate cultures.

Table 1. Metabolic parameters in control and PAN-nephropatic rats receiving vehicle (PAN) or 2-OHE (PAN+2OHE)

Parameter		Weeks of Treatment				2F-ANOVA Treatment Effect
		0	3	6	11	
Body weight (g)	Control	287 ± 3	343 ± 5	382 ± 6	421 ± 5	a: $P < 0.001$
	PAN	293 ± 4	313 ± 1^e	337 ± 15^e	382 ± 27^e	
	PAN+2OHE	291 ± 5	281 ± 5^{ef}	330 ± 8^{ef}	317 ± 13^{ef}	b: $P < 0.05$
Food intake (g/kg per d)	Control	80 ± 3	60 ± 2	60 ± 7	43 ± 3	a: $P < 0.05$
	PAN	75 ± 2	74 ± 4	69 ± 3	53 ± 5	
	PAN+2OHE	76 ± 2	75 ± 4	60 ± 5	58 ± 6	
Fluid intake (ml/kg per d)	Control	141 ± 6	107 ± 3	95 ± 7	85 ± 8	a: $P < 0.001$
	PAN	128 ± 9	182 ± 16^e	166 ± 13^e	159 ± 17^e	
	PAN+2OHE	129 ± 6	169 ± 16^e	142 ± 12^e	147 ± 14^e	
Urine volume (ml/kg per d)	Control	41.9 ± 3.7	39.4 ± 4.2	39.5 ± 5.3	28.9 ± 2.4	a: $P < 0.001$
	PAN	42.7 ± 6.6	100.6 ± 11.1	93.7 ± 12.3	113.1 ± 16.6^e	
	PAN+2OHE	40.3 ± 4.0	86.9 ± 7.0	71.8 ± 7.0	82.6 ± 7.5^e	
Sodium excretion (mEq/d per kg)	Control	4.20 ± 0.49	3.42 ± 0.32	3.12 ± 0.20	2.47 ± 0.33	c: $P < 0.03$
	PAN	4.48 ± 0.95	3.80 ± 0.58	3.92 ± 0.38	4.18 ± 0.58^e	
	PAN+2OHE	3.35 ± 0.18	5.76 ± 0.57^e	4.21 ± 0.43	3.12 ± 0.66	
Potassium excretion (mEq/d per kg)	Control	9.76 ± 0.80	9.52 ± 0.61	8.05 ± 0.66	6.88 ± 0.77	
	PAN	10.9 ± 0.72	10.7 ± 0.82	8.39 ± 0.61	9.63 ± 1.60	
	PAN+2OHE	7.54 ± 0.52	12.3 ± 0.61	9.17 ± 0.63	8.36 ± 1.25	
Creatinine excretion (mg/d)	Control	14.7 ± 1.8	12.3 ± 0.5	12.6 ± 0.6	15.8 ± 1.4	a: $P < 0.001$
	PAN	14.9 ± 2.5	11.1 ± 1.1	8.8 ± 0.9^e	18.7 ± 3.0	
	PAN+2OHE	13.0 ± 1.0	10.9 ± 0.7	9.8 ± 0.7^e	12.8 ± 2.8	
FE− Na+ (%)	Control	0.19 ± 0.04	0.20 ± 0.02	0.22 ± 0.02	0.24 ± 0.02	
	PAN	0.13 ± 0.01	0.37 ± 0.06	0.71 ± 0.13^e	1.18 ± 0.35^e	b: $P < 0.001$
	PAN+2OHE	0.18 ± 0.03	0.50 ± 0.10	0.34 ± 0.04	0.37 ± 0.04	d: $P < 0.001$
FE− K+ (%)	Control	13.0 ± 2.1	11.4 ± 0.9	12.1 ± 1.3	18.5 ± 1.5	a: $P < 0.001$
	PAN	10.5 ± 0.7	27.3 ± 3.7^e	30.1 ± 4.6^e	47.1 ± 7.7^e	
	PAN+2OHE	11.6 ± 1.4	32.1 ± 4.4^e	17.3 ± 2.5^e	29.7 ± 1.7^e	b: $P < 0.001$
Plasma triglycerides (mg/ml)	Control	115 ± 26	78 ± 19	119 ± 25	96 ± 16	a: $P < 0.001$
	PAN	117 ± 15	465 ± 90^e	531 ± 139^e	722 ± 247^e	
	PAN+2OHE	119 ± 17	552 ± 127^e	413 ± 56^e	809 ± 130^e	
Plasma cholesterol (mg/dl)	Control	53.8 ± 4.1	46 ± 3.1	51.1 ± 4.4	47.6 ± 4.2	a: $P < 0.001$
	PAN	53.6 ± 2.3	353 ± 39.4^e	283.5 ± 39.5^e	349.6 ± 43.0^e	
	PAN+2OHE	51.2 ± 2.2	315 ± 39.3^e	248.3 ± 19.3^e	340.6 ± 27.3^e	

2F-ANOVA (treatment effect): a, Control *versus* nephropathic animals; b, PAN *versus* PAN+2OHE; c, Control *versus* PAN+2OHE; d, Control *versus* PAN. Fisher's LSD *t* test: [e] *versus* Control group; [f] *versus* PAN group.

In Vivo Studies in Chronic Puromycin-Aminonucleoside Nephropathy in Rats

Animals Treatment and Experimental Protocol. A total of thirty-five, male Sprague Dawley rats (290 ± 2 g) were used in this study. Rats were housed in the University of Pittsburgh Medical Center animal care facility (temperature, 22°C; light cycle, 12 h; relative humidity, 55%). Animals were fed Pro Lab RMH 3000 rodent diet (PMI Nutrition Inc., St Louis, MO) and were given water *ad libitum*. Institutional guidelines for animal welfare were followed, and the Institutional Animal Care and Use Committee approved experimental protocols.

Before initiating the treatment and 3, 6, and 11 wk into the treatments, animals were placed in metabolic cages and allowed to acclimatize for 2 d, before conducting the 24 h measurements of urine volume, food and water intakes, and urinary sodium, potassium, creatinine, and protein excretion. Tail vein blood samples were also

taken for measurement of plasma sodium, potassium, creatinine, cholesterol, and triglyceride concentrations. Plasma and urine samples were analyzed for sodium and potassium and creatinine concentrations using a flame photometer (Model IL-943; Instrumentations Laboratory Inc., Lexington, MA) and a creatinine analyzer (Creatinine Analyzer 2; Beckman Instrument, Inc., Fullerton CA), respectively. Total urine proteins were measured by a spectrophotometric assay using bicinchoninic acid reagent (Pierce, Rockford, IL) and a modification of Lowry method (21). Plasma samples were analyzed in duplicates for triglycerides and cholesterol levels (Sigma Diagnostics, St Louis, MO).

After baseline metabolic parameters were measured, animals were randomly assigned to receive subcutaneously 3 ml/kg saline (control group, $n = 9$) or 75 mg/kg puromycin-aminonucleoside (nephropathic animals, $n = 26$). Injections of PAN (20 mg/kg) were repeated after 2, 4, 8, and 10 wk of treatment. Three hours after initial PAN
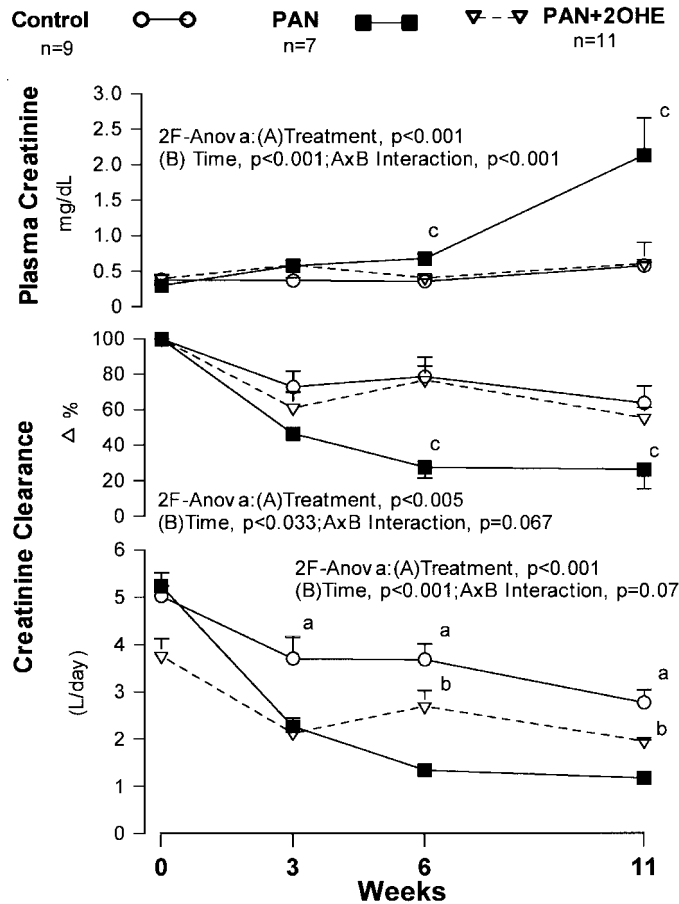
Figure 2. Plasma creatinine and creatinine clearance in control animals and in chronic puromycin aminonucleoside (PAN)–nephropathic rats treated with vehicle (PEG 400, 2.5 μl/h) or 2-hydroxyestradiol (2-OHE, 10 μg/kg per h). [a]$P < 0.05$ Control *versus* PAN and PAN+2OHE; [b]$P < 0.05$ PAN *versus* PAN+2OHE; [c]$P < 0.05$ PAN *versus* Control and PAN+2OHE.

Figure 3. Urinary protein excretion (UPE) in control animals and in chronic PAN-nephropathic rats treated with vehicle (PEG 400, 2.5 μl/hour) or 2-OHE (10 μg/kg per h). [a]$P < 0.05$ Control *versus* PAN and PAN+2OHE; [b]$P < 0.05$ PAN *versus* PAN+2OHE.

injections, control animals were implanted with osmotic minipumps (model 2ML4; Alzet, Palo Alto, CA) containing vehicle (polyethylene glycol 400, 2.5 μl/h), whereas nephropathic animals were implanted with osmotic minipumps containing vehicle (PAN group, $n = 13$) or
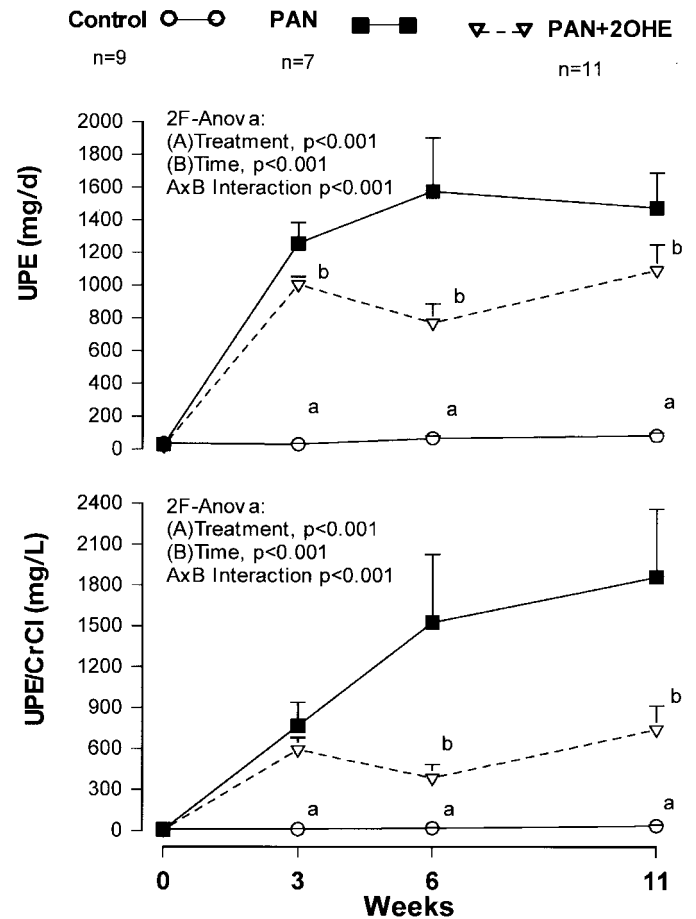
2-hydroxyestradiol (10 μg/kg per h, PAN+2OHE group, $n = 13$). Assignment to the PAN and PAN+2OHE groups was random. Six animals in PAN group and two animals in PAN+2OHE groups died during the treatment, and these animals were not used in the final data analysis.

Table 2. Acute measurements (mean $\pm$ SEM) of renal hemodynamic and excretory function in control animals (Control) and in PAN-nephropathic animals receiving vehicle (PAN) or 2-hydroxyestradiol (PAN+2OHE) for 12 wk

Parameters	Control	PAN	PAN+2OHE
Body weight	433 ± 5[a]	368 ± 15	353 ± 15
Left kidney (g)	1.44 ± 0.2[a]	2.10 ± 0.13	2.34 ± 0.09
Mean BP (mmHg)	133.4 ± 2.3	153.0 ± 6.1	138.2 ± 4[b]
Renal blood flow (ml/min per g kidney)	5.03 ± 0.28[a]	2.73 ± 0.24	2.80 ± 0.18
Hematocrit (%)	50 ± 1[a]	27 ± 2	30 ± 3
Renal plasma flow (ml/min per g kidney)	2.5 ± 0.12	2.00 ± 0.19	1.96 ± 0.12
Renal vascular resistance (mmHg/ml per min per g kidney)	28.3 ± 1.26[a]	63.6 ± 7.6	58.5 ± 4.5
Urine volume (μl/min per g kidney)	5.7 ± 0.6	4.43 ± 0.85	3.33 ± 0.26
Glomerular filtration rate (ml/min per g kidney)	1.65 ± 0.12[a]	0.21 ± 0.07	0.54 ± 0.06[b]

2F-ANOVA, $P < 0.05$; [a] Control *versus* nephropathic animals; [b] PAN *versus* PAN+2OHE.

Figure 5. Assessment of glomerular immunohistochemical staining for PCNA of control (CONT), PAN-nephropathic rats (PAN), and PAN-nephropathic rats treated with 2-hydroxyestradiol (PAN+2-OHE). Staining was assessed by quantitative image analysis with a SAMBA 4000 image analyzer as described in the Materials and Methods section. Results represent mean $\pm$ SEM of the labeling index.

Figure 4. Representative proliferating cell nuclear antigen (PCNA) immunohistochemistry of cortical sections from PAN-nephropathic rat (A), PAN-nephropathic rat treated with 2-OHE (B), and control rat (C). Magnification, $\times 400$.

At 12 wk into treatment, animals were anesthetized with pentobarbital (45 mg/kg intraperitoneally) and instrumented for measurements of renal hemodynamics and excretory function. Two PE-50 catheters were inserted into left jugular vein for delivery of supplemental anesthetics and saline infusion (50 μl/min), respectively. Another PE-50 catheter was inserted into left carotid artery and connected to a BP analyzer (Micro-Med., Inc) for continuous measurement of BP and heart rate. A PE-10 catheter was inserted into the left ureter to facilitate collection of urine, and a flow probe (Model 1RB; Transonic Systems, Inc., Ithaca, NY) was placed on the left renal artery for
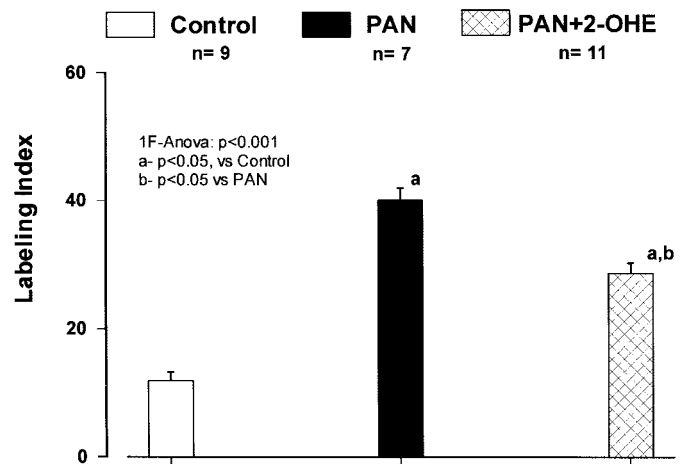
determination of renal blood flow (RBF), which was used to calculate renal vascular resistance (RVR). Next, an infusion of ^{14}C-inulin (0.035 μCi/20 μl of saline per min) was initiated, and after 60 min two 30-min clearance periods were conducted. A midpoint blood sample (300 μl) was collected, and plasma and urine ^{14}C-inulin radioactivity was measured. Renal clearance of ^{14}C-inulin was calculated as an estimate of GFR.

An aliquot of midpoint blood sample drawn during the first clearance period was used for testosterone measurements. Plasma testosterone levels were determined by RIA using a commercial kit provided by ICN Biomedicals (Costa Mesa, CA) according the protocol of the manufacturer.

Renal Histopathology and Immunohistochemical Studies. Animals were euthanatized by anesthetic overdose, and kidneys were removed and weighed. Right kidney was fixed in 10% formalin buffer for subsequent light microscopy and immunohistochemistry. The kidney tissue sample was sectioned and processed into paraffin blocks for light microscopy. Five-micron tissue sections from formalin-fixed, paraffin-embedded renal cortices were dewaxed and stained with periodic acid-Schiff (PAS) stain for histologic assessment. Kidney slices were examined by light microscopy and scored in a blinded fashion by one of the investigators (E.S.). Histopathologic features were assessed semiquantitatively on 10 high power fields ($\times 400$) and included segmental (FSGS) and global (FGGS) glomerulosclerosis, tubular atrophy (0 to 3^+), interstitial inflammation (0 to 3^+), tubular dilation (0 to 4^+), arterial medial hypertrophy, and arteriolar sclerosis (0 to 3^+).

Renal cortical segments (5 μm) were incubated for 1 h at room temperature with polyclonal pan-specific transforming growth factor–β (TGF-β) anti- rabbit antibody (1:50 dilution; R&D Systems, Minneapolis, MN). For labeling of collagen IV, samples were incubated overnight at 4°C with rabbit anti-mouse collagen IV antibody (dilution 1:500) obtained from Chemicon International Inc. (Temecula, CA). A primary monoclonal mouse antibody (1:200 dilution; Dako, Carpenteria, CA) was used to label proliferating cell nuclear antigen (PCNA). A rat ED1 antibody (Serotec, Raleigh, NC) specific
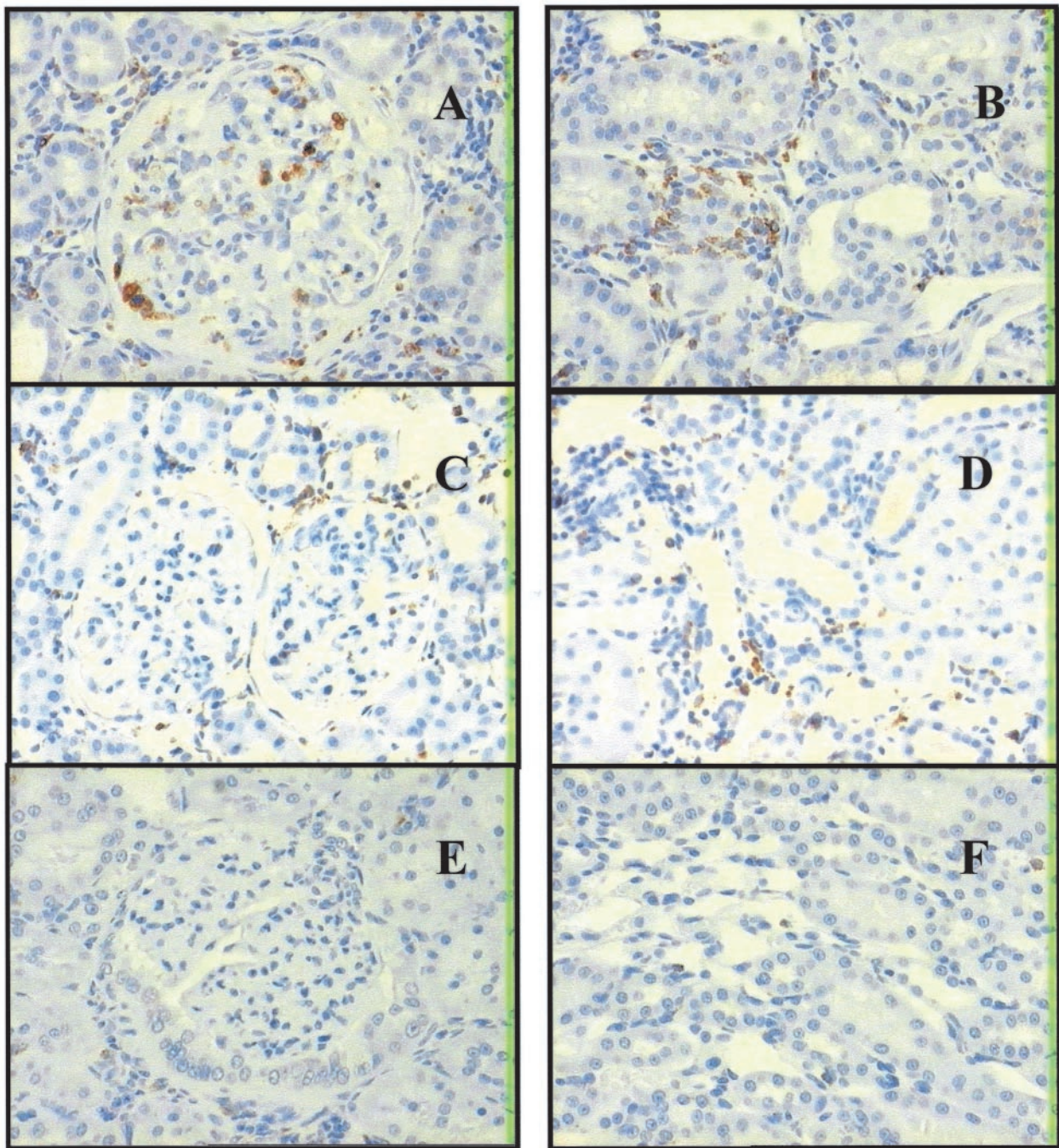
Figure 6. Representative immunohistochemistry of cortical sections for ED1$^+$ cells in glomeruli and interstitium of PAN-nephropathic rat (A and B), PAN-nephropathic rat treated with 2-OHE (C and D), and control animal (E and F). Magnification, ×400.

for a monocyte/macrophage cytoplasmatic antigen was used to label glomerular and interstitial macrophages. Nonspecific staining was assessed by replacing the primary antibody with PBS. Sections were washed and further developed according to the directions of the manufacturer (Dako) using the LSAB2 kit that contained a second antibody linked to avidin and peroxidase-conjugated biotin. Immuno-chemical staining for TGF-β, collagen IV, and PCNA were assessed quantitatively with a SAMBA 4000 image analyzer (Image Products International, Chantilly, VA) using specialized computer software (Immuno-Analysis, version 4.1; Microsoft, Richmond, WA), a color video camera and a Compaq computer. Software designed for immu-nostaining analyses enabled the operator to set density threshold values by averaging several fields on the negative control tissues in which the primary antibody was replaced with PBS. Background subtraction was then performed automatically on every tissue. Ten high power fields (×400) were assessed for staining density or pos-itively marked cells for ED-1. The results are reported as the labeling index, which represents the percentage of the total examined area that
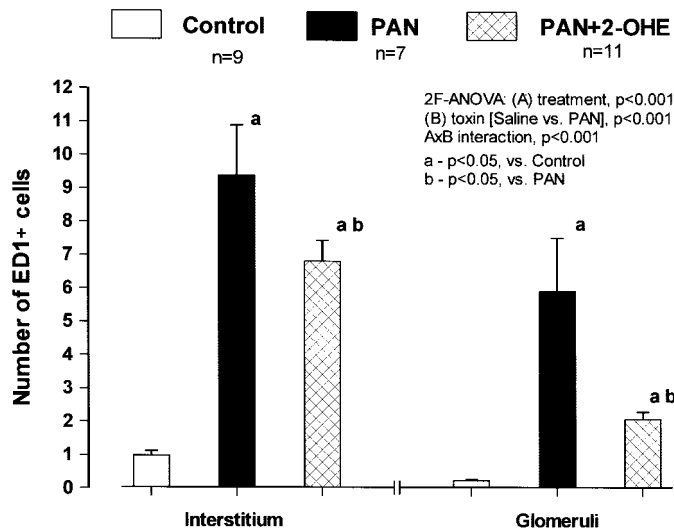
Figure 7. Assessment of glomerular immunohistochemical staining for ED1$^+$ cells in glomeruli and interstitium of control animals (CONT), PAN-nephropathic rats (PAN), and PAN-nephropathic rats treated with 2-hydroxyestradiol (PAN+2-OHE). Staining was assessed by quantitative image analysis with a SAMBA 4000 image analyzer as described in the Materials and Methods section. Results represent mean ± SEM of the labeling index.

stained positively. Staining intensity of positive areas was also assessed (mean optical density), and a mean quick score was then calculated (mean optical density × labeling index).

Statistical Analyses. All data are presented as mean ± SEM. Statistical analyses were performed using the Number Cruncher Statistical software program (Kaysville, Utah). Group comparison for data from metabolic studies (repeated measurements) were performed by one (1F) and two (2F) hierarchical ANOVA as appropriate, followed by Fisher's LSD test for *post hoc* comparison. Comparison of data from acute experiments and from histologic analyses (single point data) was performed by 1F-ANOVA (all three groups) or *t* test (PAN *versus* PAN+2OHE). The probability value of $P < 0.05$ was considered statistically significant.

Results

The effects of 2-hydroxyestradiol on rat mesangial cells growth, proliferation, and collagen production are presented in Figure 1. 2-OHE inhibited serum (2.5%)–induced cell growth in a concentration-dependent manner as assessed by ^{3}H-thymidine incorporation (Figure 1, upper panel). Similarly, 2-OHE produced significant (1F-Anova, $P < 0.001$) and concentration-dependent inhibition of serum-induced collagen synthesis (^{3}H-proline incorporation; Figure 1, middle panel) and cell proliferation (cell number; Figure 1, bottom panel). Importantly, the inhibitory effects of 2-OHE (0.1 μmol/L) on ^{3}H-thymidine and ^{3}H-proline incorporation were not blocked by a 500-times higher concentration of the selective estrogen receptor antagonist ICI 182780 (Figure 1, top and middle panels). The latter suggests that the inhibitory effect of 2-OHE on mesangial cell activity was not mediated by estrogen receptors.

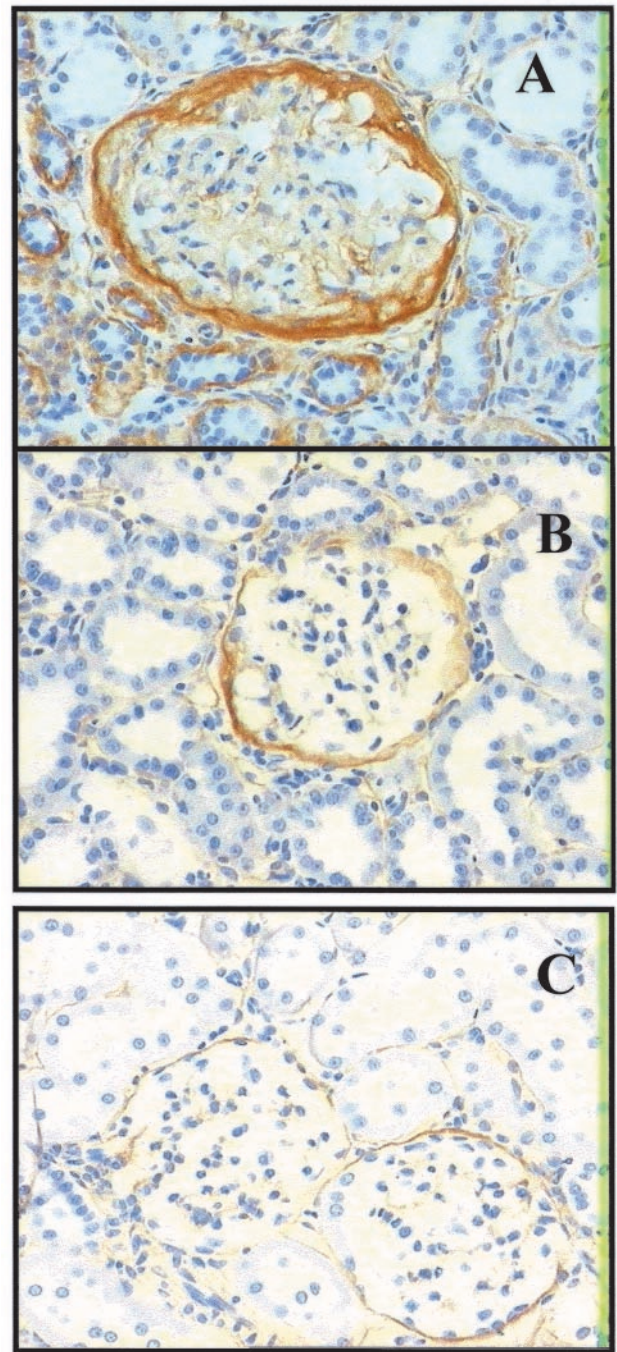Chronic administration of PAN resulted in severe renal

Figure 8. Representative collagen IV immunochemistry of cortical sections from PAN-nephropathic rat (A), PAN-nephropathic rat treated with 2-OHE (B), and control rat (C). Magnification, ×400.

damage. In nephropathic animals, PAN induced severe proteinuria, increased plasma creatinine levels, reduced creatinine clearance, and increased plasma cholesterol and triglycerides levels. Nephropathic animals also had reduced body weight and increased food and fluid intake, urine output, and fractional excretion of sodium and potassium (Table 1). Chronic treatment with 2-OHE attenuated PAN-induced increase in plasma creatinine levels (Figure 2, top panel) and attenuated the decline in creatinine clearance in nephropathic animals (Figure 2,
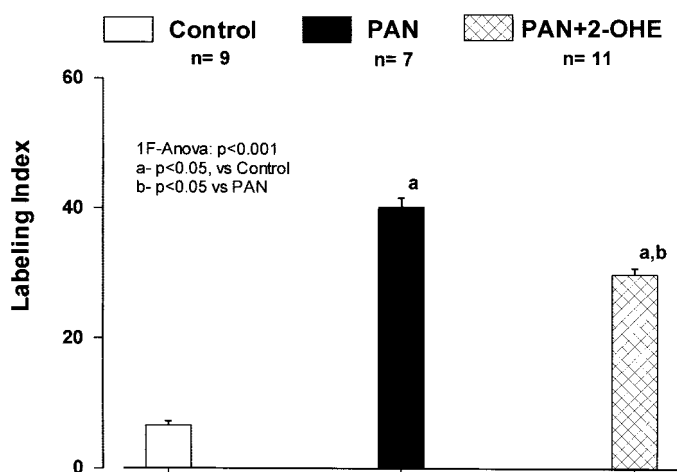
Figure 9. Assessment of glomerular immunohistochemical staining for collagen IV of control animals (CONT), PAN-nephropathic rats (PAN), and PAN-nephropathic rats treated with 2- hydroxyestradiol (PAN+2-OHE). Staining was assessed by quantitative image analysis with a SAMBA 4000 image analyzer as described in the Materials and Methods section. Results represent mean ± SEM of the labeling index.

middle and bottom panels). Furthermore, 2-OHE reduced PAN-induced increase in urinary protein excretion (UPE; Figure 3, top panel), and this effect became even more significant when UPE was corrected for reduction in glomerular excretory function (*i.e.*, creatinine clearance; Figure 3, bottom panel). More importantly, 2-OHE reduced mortality by 66%.

Acute measurements of renal hemodynamics and excretory function (Table 2) revealed increased BP and renal vascular resistance and decreased renal blood flow, hematocrit, and GFR (inulin clearance) in nephropathic rats as compared with control rats (Table 2). Importantly, 2-OHE abolished PAN-induced changes in BP and significantly attenuated PAN-induced changes in GFR.

PAN-nephropathic animals tended to have lower testosterone levels compared with control rats with intact kidneys; more importantly, treatment with 2-OHE did not lower testosterone levels (Cont, 1.49 ± 0.2; PAN, 0.92 ± 0.27; PAN+2OHE, 0.91 ± 0.22 ng/ml).

Light microscopy revealed severe glomerulosclerosis, significant tubular dilation, and atrophy with presence of protein casts and interstitial inflammation and fibrosis in PAN-nephropathic animals. Furthermore, immunohistochemical stainings for collagen IV, PCNA, ED1^{+} cells, and TGF-β were significantly higher in PAN-nephropathic rats *versus* control animals with intact kidneys. Treatment with 2-OHE had no effect in renal tubular cells on increased TGF-β content (data not shown). Representative examples of glomerular staining for PCNA in renal cortical sections from each study group are shown in Figure 4, and assessment of glomerular staining by quantitative image analysis is shown in Figure 5. As assessed by quantitative analysis, the area positive for PCNA staining (labeling index, Figure 5) was markedly expanded in glomeruli from PAN-nephropathic rats compared with control animals

with intact kidneys. Treatment with 2-OHE significantly reduced the labeling index of glomerular immunoreactive PCNA in PAN-nephropathic animals.

Figures 6 and 7 illustrate the influence of 2-OHE on glomerular and interstitial macrophage infiltration. PAN induced a several-fold increase in the number of ED1^{+} cells both in glomeruli and in the interstitium. Importantly, treatment with 2-hydroxestradiol significantly ($P < 0.001$) reduced the number of inflammatory cells in glomeruli and in the interstitium of nephropathic kidneys (Figure 7). Representative examples of glomerular staining for collagen IV in cortical glomeruli from each study group are shown in Figure 8. The significant increase in glomerular collagen IV content was detected in PAN-nephropathic rats (Figure 8A) compared with control animals with intact kidneys (Figure 8C). Treatment with 2-OHE significantly decreased PAN-induced increase in collagen IV (Figure 8B) and significantly reduced the labeling index of glomerular immunoreactive collagen IV in PAN-nephropathic animals (Figure 9).

Discussion

Similar to the progression of cardiovascular disease, there is a plethora of clinical data suggesting that, when adjusted for demographic and other factors for renal disease, women are more resistant to and have slower progression of renal disease than men. This finding suggests that estrogens are renoprotective and that, therefore, hormone replacement therapy would be a rational approach to attenuate chronic renal failure (CRF) in postmenopausal women with renal disease. However, because estrogenic activity increases the risk of cancer, this approach would be of limited value in premenopausal women and in men with CRF. Moreover, a recent large clinical study (HERS) has questioned the clinical benefits of conjugated estrogens in prevention of cardiovascular disease in woman (22).

Our recent *in vitro* data suggest that the catechol metabolites of 17-β estradiol (*i.e.*, 2-OHE) may provide greater cardiovascular and renal protection than estradiol and that these effects are not mediated by estrogen receptors (5–8). Therefore, very recently we conducted a study with 2-OHE in male, obese (fa-fa[cp]), diabetic ZSF1 rats, a genetic model of obesity and the metabolic syndrome, with a high risk for renal disease (13,14). Chronic administration of 2-OHE provided significant renoprotection, as evidence by decreased proteinuria, glomerulosclerosis, and severity of tubulointerstitial changes (15,16). However, 24-wk treatment with 2-OHE induced a significant reduction in food consumption and body weight, significantly decreased elevated plasma cholesterol levels, and improved glucose control; it is therefore possible that the renoprotective effects of 2-OHE were due to the improved metabolic status rather that to direct renal effects. Therefore, we examined the potential direct renoprotective effects of 2-hydroxestradiol, both *in vitro* and *in vivo*.

In vitro, in rat GMC in culture, 2-OHE inhibited collagen synthesis, DNA synthesis, and cell proliferation. This is in accordance with the known effects of estradiol, which has been shown to inhibit serum-induced collagen synthesis (23,24) and collagen synthesis induced by angiotensin II, endothelin-1, and

TGF-β (24–26). Furthermore, estradiol also inhibits GMC growth (27) as well as the production of growth promoters that induce glomerulosclerosis, such as angiotensin I and endothelin-1 (28,29). Importantly, in the present study, the inhibitory effects of 2-OHE (0.1 μmol/L) were not affected by a 500-times higher concentration of a highly specific estrogen receptor antagonist ICI 182780. These data strongly suggest that 2-OHE may provide renoprotective effects by inhibiting some of the key proliferative mechanisms of glomerulosclerosis, and this inhibition is not mediated by estrogen receptors.

Indeed, *in vivo* in chronic PAN nephropathy, a model that resembles human FSGS, 2-OHE provided significant renoprotection. Continuous administration of nonestrogenic doses (9) of 2-OHE slowed the progression and reduced the severity of nephropathy and histopathologic changes in male nephropathic rats. In this regard, immunohistochemical analyses revealed reduced macrophage influx in PAN-nephropathic animals treated with 2-OHE. It is not clear whether the reduced interstitial inflammation was a primary effect of 2-OHE or was due to the 2-OHE-induced reduction in UPE. Reduced proteinuria and subsequent reduction in protein leakage into interstitium would be expected to attenuate interstitial events, including interstitial inflammation.

Immunohistochemical staining for glomerular PCNA revealed increased mesangial cell proliferative activity in PAN-nephropathic rats (Figure 4). Importantly, 2-OHE significantly (Figure 5, $P < 0.001$) attenuated proliferative processes in glomeruli in PAN-nephropathic rats. The reduced proliferative activity *in vivo* in 2-OHE–treated rats substantiates previously observed *in vitro* effects of 2-OHE in rat mesangial cells. To the best of our knowledge, this is the first study to report both *in vitro* and *in vivo* antiproliferative effects of 2-OHE in mesangial cells. Taken together, the findings confirm our previous results in male obese ZSF1 rats (15) and suggest that 2-OHE may be safe and effective in the male gender. Moreover, our findings indicate that the renoprotective effects of 2-OHE are independent of its effects on metabolic status.

In cell culture experiments, significant inhibition (−20 to −30%) of mesangial cell proliferation and collagen accumulation (Figure 1) occurred at concentrations as low as 1 nmol/L or 288 pg/ml of 2-OHE. Similar plasma concentrations (250 to 260 pg/ml) have been reported in rats after chronic infusion of approximately 180 μg/kg per d of 2-OHE (12). This dose is comparable with the dose of 2-OHE (*i.e.*, 240 μg/kg per d) administered to PAN-nephropathic rats. Little is known regarding the physiologic concentrations of 2-OHE in women and men. During the menstrual cycle, estradiol concentrations fluctuate (60 to 300 pg/ml) with an average cycle concentration of approximately 150 pg/ml (30), whereas estradiol concentrations in men are steady and roughly 40 to 50 pg/ml (31). It is difficult to estimate circulating levels of 2-OHE from known concentrations of estradiol. Nonetheless, the expected plasma concentrations of 2-OHE in the present study should be only moderately above the physiologic plasma concentrations of 2-OHE in women. Finally, it is not clear whether the observed renoprotective effects in PAN-nephropathic animals could be entirely ascribed to 2-OHE. This major estrogen metabolite is

cleared from plasma 10 times faster than 17β-estradiol (12). In plasma, 2-OHE undergoes fast conversion to 2-methoxyestradiol (by catechol O-methyltransferase [COMT] derived from erythrocytes), and has a very short half-life of 60 to 90 s (32). Therefore, further studies are warranted to determine whether the observed renoprotective effects are due to 2-OHE, 2-methoxyestradiol, or both.

An important finding of this study that merits further elaboration is the neutral effect of 2-OHE on plasma cholesterol and triglycerides levels. Chronic administration of PAN induced a several-fold increase in plasma lipids, a finding consistent with the well-known effects of nephropathy in rats to decrease triglyceride clearance and increase cholesterol synthesis (33,34). In the present study, 2-OHE did not reduce the increased plasma cholesterol levels. This contrasts with our previous study in obese diabetic ZSF1 rats, in which 2-OHE significantly reduced elevated cholesterol levels (15), and with the study of Liu and Bachmann (10), who demonstrated in ovarectomized rats that 2-OHE exerted a significant hypocholesterolemic effect with no estrogenic (*i.e.*, uterotropic) effects. It is not clear why 2-OHE did not reduce elevated cholesterol levels in the present study. Nevertheless, 2-OHE exhibited renoprotection, despite no effect on elevated cholesterol levels. Therefore, an important implication of the present study is that the renoprotective effects of 2-OHE are at least in part independent of changes in lipid status.

Importantly, similar to our previous study in obese diabetic ZSF1 rats (15), 2-OHE had no effect on elevated triglyceride levels in the present study. Estradiol has been shown to increase plasma triglyceride levels by increasing triglyceride production and secretion (35,36). This effect may be particularly important in the nephrotic syndrome, where hyperlipidemia is due to reduced triglyceride clearance. In this setting, estrogen may further elevate plasma triglycerides and further increase the risk for cardiovascular and renal disease. Indeed, estrogens increase triglyceride levels in adriamycin-induced nephrotic syndrome (37), worsen incipient hypertriglyceridemia and accelerate the development of renal disease in obese Zucker rats (38,39), and cause a further increase in triglyceride and cholesterol levels and induce glomerulosclerosis in analbuminemic rats (40). These findings suggest that estrogens may be contraindicated in subjects with nephrotic syndrome who regularly have hypoalbuminemia and hypertriglyceridemia. It is important to note that our previous (14) and current studies indicate that 2-OHE does not alter triglycerides levels and therefore may be safe and renoprotective in nephrotic syndrome associated with hypertriglyceridemia. However, further studies in female rats with nephrotic proteinuria and hypertriglyceridemia are warranted to clarify whether 2-OHE has neutral effects on elevated lipids in female rats with nephrotic syndrome.

2-OHE exhibits some (although very low) estrogenic activity, and androgens have been suggested to accelerate chronic renal failure (41,42); it is therefore possible that at least part of the renoprotective effect of 2-OHE was due to its estrogenic activity and subsequent reduction in testosterone levels. Importantly, 12-wk treatment with 2-OHE did not change plasma

testosterone levels in nephropathic male rats in the present study.

In summary, this study provides the first evidence that 2-OHE, a metabolite of estradiol with little estrogenic activity, attenuates the progression of renal failure in experimental nephropathy that resembles FSGS. The renoprotective effect is due most likely to the inhibition of some of the key proliferative mechanisms involved in glomerular remodeling and glomerulosclerosis, and this inhibition is not mediated by estrogen receptors.

Acknowledgments

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