2-Hydroxyestradiol slows progression of experimental polycystic kidney disease

Sharon Anderson, Terry T. Oyama, Jessie N. Lindsley, William E. Schutzer, Douglas R. Beard

Division of Nephrology and Hypertension, Department of Medicine, Oregon Health & Science University, Portland; Medical and Research Services, Portland Veterans Affairs Medical Center, Portland, Oregon; and Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Indiana

Submitted 16 May 2011; accepted in final form 2 December 2011

2-Hydroxyestradiol slows progression of experimental polycystic kidney disease. Am J Physiol Renal Physiol 302: F636–F645, 2012. First published December 7, 2011; doi:10.1152/ajprenal.00265.2011.—Male gender is a risk factor for progression of polycystic kidney disease (PKD). 17β-Estradiol (E2) protects experimentally, but clinical use is limited by adverse effects. Novel E2 metabolites provide many benefits of E2 without stimulating the estrogen receptor, and thus may be safer. We hypothesized that E2 metabolites are protective in a model of PKD. Studies were performed in male control Han:SPRD rats, and in cystic males treated with orchiectomy, 2-methoxyestradiol, 2-hydroxyestradiol (2-OHE), or vehicle, from age 3 to 12 wk. Cystic rats exhibited renal functional impairment (~50% decrease in glomerular filtration and renal plasma flow rates, $P < 0.05$) and substantial cyst development (20.5 ± 2.0% of cortex area). 2-OHE was the most effective in limiting cysts (6.0 ± 0.7% of cortex area, $P < 0.05$ vs. vehicle-treated cystic rats) and preserving function, in association with suppression of proliferation, apoptosis, and angiogenesis markers. Downregulation of p21 expression and increased expression of Akt, the mammalian target of rapamycin (mTOR), and some of its downstream effectors were significantly reversed by 2-OHE. Thus, 2-OHE limits disease progression in a cystic rodent model. Mechanisms include reduced renal cell proliferation, apoptosis, and angiogenesis. These effects may be mediated, at least in part, by preservation of p21 and suppression of Akt and mTOR. Estradiol metabolites may represent a novel, safe intervention to slow progression of PKD.

gender; estrogen; mammalian target of rapamycin 2; hydroxyestradiol p21; hypoxia-inducible factor-1α

More promising are novel estrogen metabolites, such as 2-methoxyestradiol (2-ME) and its precursor 2-hydroxyestradiol (2-OHE). These compounds share many beneficial actions of 17β-estradiol (E2), but they act independently of estrogen receptors (11). E2 metabolites are protective in several renal disease models including insulin resistance (60, 75), puromycin nephrosis (59), and nitric oxide synthase (NOS) inhibition-induced injury (61).

In PKD, somatic gene mutations induce an environment with increased activity of growth factors, cytokines, and other mediators, which then induce renal tubular epithelial (RTE) cell proliferation, apoptosis, fluid secretion, interstitial inflammation, and extracellular matrix (ECM) accumulation (24, 63, 70). A role for angiogenesis has also been suggested (2, 68). Whereas E2 is mitogenic, proangiogenic, and anti-apoptotic, 2-OHE and 2-ME are anti-mitogenic, anti-angiogenic, and proapoptotic (11, 37). These attributes make the metabolites particularly attractive for study in PKD.

Accordingly, we explored the potential neproprotective effect of 2-ME and 2-OHE in the Han:SPRD rat model of ADPKD and potential mechanisms of protection. We hypothesized that E2 metabolites would protect against cyst growth and loss of renal function in polycystic rats and that protective mechanisms include effects on cell proliferation, apoptosis, and/or angiogenesis. We further examined effects on some known signaling mediators that control cell proliferation, growth, and protein synthesis in PKD.

MATERIALS AND METHODS

Animals. Studies were conducted in male Han:SPRD rats, from a colony derived from rats provided by Benjamin D. Cowley, Jr. (then at the Univ. of Kansas Medical Center, Kansas City, KS). Heterozygous cystic (Cy+/+) and unaffected littermate control (+/+ ) rats were used. Rats were fed standard rat chow (Rodent Laboratory Chow 5010; Purina Mills, Richmond, IN) ad libitum with free access to water. The Portland Veterans Affairs Institutional Animal Care and Use Subcommittee approved these studies, which were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male rats were weaned at 3 wk of age (WOA), at which time they were anesthetized with isoflurane for laparoscopic determination of animals. Studies were conducted in male Han:SPRD rats, from a colony derived from rats provided by Benjamin D. Cowley, Jr. (then at the Univ. of Kansas Medical Center, Kansas City, KS). Heterozygous cystic (Cy+/+) and unaffected littermate control (+/+ ) rats were used. Rats were fed standard rat chow (Rodent Laboratory Chow 5010; Purina Mills, Richmond, IN) ad libitum with free access to water. The Portland Veterans Affairs Institutional Animal Care and Use Subcommittee approved these studies, which were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male rats were weaned at 3 wk of age (WOA), at which time they were anesthetized with isoflurane for laparoscopic determination of
At 12 WOA, body weight, systolic blood pressure by the tail-cuff method, and 24-h urine collections for measurement of protein, albumin, and N-acetyl-D-glucosaminidase (NAG) were measured. One week later, renal function studies were performed, after which kidneys were taken for studies described below.

**Renal function studies.** Under inactin anesthesia (100 mg/kg ip), glomerular filtration rate (GFR; by inulin clearance) and effective renal plasma flow [ERPF; by clearance of para-aminohippurate (PAH)] were measured using standard methods (28, 30, 51). Renal vascular resistance (RVR) was calculated using standard formulas.

**Morphologic studies.** Kidneys were immersed in 10% formalin, then dehydrated through ethanols, embedded in paraffin, sectioned at 4-μm thickness, and placed onto glass slides. Cyst burden was quantified by a point counting method (21, 28) on periodic acid Schiff-stained sections.

**Immunoblotting.** The kidneys were homogenized in RIPA buffer containing protease and phosphatase inhibitors and further processed to obtain whole cell homogenates and nuclear fractions, as previously described (30, 31). To obtain nuclear fractions for hypoxia-inducible factor-1 (HIF-1), the pellet obtained from RIPA preparation was suspended and incubated on ice for 2 h with intermittent mixing. This suspension was then centrifuged at 14,000 rpm at 4°C for 30 min. The supernatant (nuclear extract) was collected and stored at -70°C until use. Normoxic and hypoxic lysate controls were obtained from Novus Biologicals (Littleton, CO). Total protein content in fractions was determined by BCA analysis (Pierce Biotechnology, Rockford, IL).

### Table 1. Systemic and renal parameters in Han:SPRD rats

<table>
<thead>
<tr>
<th>Group</th>
<th>SBP, mmHg</th>
<th>U_{protV}, mg/day</th>
<th>U_{albV}, μg/day</th>
<th>U_{NAGV}, U/day</th>
<th>BW, g</th>
<th>LKW, g</th>
<th>LKW/100, g BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>135 ± 4</td>
<td>6 ± 0.4</td>
<td>0.6 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>392 ± 10</td>
<td>1.45 ± 0.04</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>Cys/Veh</td>
<td>160 ± 7^a</td>
<td>15 ± 3^a</td>
<td>11.8 ± 2.6^b</td>
<td>2.1 ± 0.3</td>
<td>356 ± 15</td>
<td>4.10 ± 0.25^a</td>
<td>1.15 ± 0.03^a</td>
</tr>
<tr>
<td>Cys/Orch</td>
<td>135 ± 5^c</td>
<td>4 ± 1^c</td>
<td>2.7 ± 0.7^c</td>
<td>1.2 ± 0.1</td>
<td>337 ± 3^a</td>
<td>3.50 ± 0.08^a</td>
<td>1.04 ± 0.02^a</td>
</tr>
<tr>
<td>Cys/2-ME</td>
<td>148 ± 4^d</td>
<td>4 ± 1^d</td>
<td>8.7 ± 2.8^a</td>
<td>1.6 ± 0.2</td>
<td>315 ± 12^a</td>
<td>3.01 ± 0.20^c</td>
<td>0.95 ± 0.05^c</td>
</tr>
<tr>
<td>Cys/2-OHE</td>
<td>140 ± 4^e</td>
<td>1 ± 0.1^ec</td>
<td>0.6 ± 0.1^df</td>
<td>1.3 ± 0.2</td>
<td>307 ± 9^ec</td>
<td>2.22 ± 0.08^ace</td>
<td>0.72 ± 0.02^ace</td>
</tr>
</tbody>
</table>

Values are means ± SE. SBP, systolic blood pressure; U_{protV}, urinary protein excretion; U_{albV}, urinary albumin excretion; U_{NAGV}, urinary excretion of N-acetyl-D-glucosaminidase; BW, body weight; LKW, left kidney weight. ^aP < 0.05, ^bP < 0.01 vs. Control. ^cP < 0.05, ^dP < 0.01 vs. cystic rats receiving vehicle (Cys/Veh). ^eP < 0.05, 2-methoxyestradiol (2-ME) or 2-hydroxyestradiol (2-OHE) vs. cystic rats with orchiectomy (Cys/Orch). ^fP < 0.05 vs. Cys/2-ME.

Fig. 1. Cyst formation in noncystic control (Cont) rats and cystic rats receiving vehicle (Cys/Veh), cystic rats with orchiectomy (Cys/Orch), and cystic rats treated with 2-methoxyestradiol (2-ME) or 2-hydroxyestradiol (2-OHE; each n = 8–14). Cyst burden was quantified by a point counting method (21, 28) on periodic acid Schiff-stained sections.

**Immunoblotting.** The kidneys were homogenized in RIPA buffer containing protease and phosphatase inhibitors and further processed to obtain whole cell homogenates and nuclear fractions, as previously described (30, 31). To obtain nuclear fractions for hypoxia-inducible factor-1α (HIF-1α), the pellet obtained from RIPA preparation was suspended and incubated on ice for 2 h with intermittent mixing. This suspension was then centrifuged at 14,000 rpm at 4°C for 30 min. The supernatant (nuclear extract) was collected and stored at -70°C until use. Normoxic and hypoxic lysate controls were obtained from Novus Biologicals (Littleton, CO). Total protein content in fractions was determined by BCA analysis (Pierce Biotechnology, Rockford, IL).

**Morphologic studies.** Kidneys were immersed in 10% formalin, then dehydrated through ethanols, embedded in paraffin, sectioned at 4-μm thickness, and placed onto glass slides. Cyst burden was quantified by a point counting method (21, 28) on periodic acid Schiff-stained sections.

**Immunoblotting.** The kidneys were homogenized in RIPA buffer containing protease and phosphatase inhibitors and further processed to obtain whole cell homogenates and nuclear fractions, as previously described (30, 31). To obtain nuclear fractions for hypoxia-inducible factor-1α (HIF-1α), the pellet obtained from RIPA preparation was suspended and incubated on ice for 2 h with intermittent mixing. This suspension was then centrifuged at 14,000 rpm at 4°C for 30 min. The supernatant (nuclear extract) was collected and stored at -70°C until use. Normoxic and hypoxic lysate controls were obtained from Novus Biologicals (Littleton, CO). Total protein content in fractions was determined by BCA analysis (Pierce Biotechnology, Rockford, IL).

**Morphologic studies.** Kidneys were immersed in 10% formalin, then dehydrated through ethanols, embedded in paraffin, sectioned at 4-μm thickness, and placed onto glass slides. Cyst burden was quantified by a point counting method (21, 28) on periodic acid Schiff-stained sections.

**Immunoblotting.** The kidneys were homogenized in RIPA buffer containing protease and phosphatase inhibitors and further processed to obtain whole cell homogenates and nuclear fractions, as previously described (30, 31). To obtain nuclear fractions for hypoxia-inducible factor-1α (HIF-1α), the pellet obtained from RIPA preparation was suspended and incubated on ice for 2 h with intermittent mixing. This suspension was then centrifuged at 14,000 rpm at 4°C for 30 min. The supernatant (nuclear extract) was collected and stored at -70°C until use. Normoxic and hypoxic lysate controls were obtained from Novus Biologicals (Littleton, CO). Total protein content in fractions was determined by BCA analysis (Pierce Biotechnology, Rockford, IL).
Denatured proteins were separated through an SDS gel and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Following being blocked, membranes were incubated overnight with antibodies raised against p21Waf1/Cip1 (p21; 1:800, Santa Cruz Biotechnology, cat. no. sc-756), HIF-1α (1:300, Novus Biologicals, cat. no. NB 100-134), vascular endothelial growth factor (VEGF; 1:800, Santa Cruz Biotechnology, cat. no. sc-152), phospho-serine 473-Akt (p-Akt; 1:800, Cell Signaling, Beverly, MA, cat. no. 4058), phospho-serine 2448-mammalian target of rapamycin (mTOR; p-mTOR; 1:500, Cell Signaling, cat. no. 2971), phospho-threonine 489-p70 S6 Kinase (p-S6K; 1:500, Cell Signaling, cat. no. 9205), or phospho-serine 65-4E-BP1 (p-4E-BP1; 1:500, Cell Signaling, cat. no. 9451). Immunodetection was accomplished using goat anti-rabbit secondary antibody conjugated with horseradish peroxidase for 60 min (1:80,000, Pierce) in Tris-buffered saline/Tween 20 containing 5% nonfat dry milk. Visualization was performed with an enhanced chemiluminescence Western blotting kit (Supersignal West Dura, Pierce). After detection of phosphorylated proteins (p-Akt, p-mTOR, p-S6K, and p-4E-BP1), membranes were stripped and reincubated with rabbit polyclonal antibodies against total Akt (1:800, Cell Signaling, cat. no. 9272), total mTOR (1:1,000, Cell Signaling, cat. no. 2971), total S6K (p-S6K; 1:500, Cell Signaling, cat. no. 9205), or total 4E-BP1 (1:800, Cell Signaling, Beverly, MA, cat. no. 4058), and processed as described above. To confirm equality of loading, all membranes were stripped and reanalyzed for actin expression (Santa Cruz Biotechnology).

**Immunohistochemistry.** The left kidney was fixed in 10% formalin and embedded in paraffin. Four-micrometer sections were deparaffinized in xylene and rehydrated through graded ethanols to water, and pretreated by steaming in 10% CITRA buffer (BioGenex, San Ramon, CA). After being blocked, slides were incubated overnight at 4°C with primary antibody to nuclear antigen (PCNA; FL-261, 1:500, Santa Cruz Biotechnology), p21 (H-164, 1:500, Santa Cruz Biotechnology), or proliferating cell nuclear antigen (PCNA; FL-261, 1:500, Santa Cruz Biotechnology), with the same concentration of nonimmune rabbit IgG as a control. Endogenous peroxidase activity was blocked with 3% H2O2 solution in methanol. The primary antibody was localized using the Vectastain Elite ABC peroxidase detection system (Vector Laboratories, Burlingame, CA). This was followed by reaction with diaminobenzidine (Dajindo, Japan) as chromogen and counterstaining with Harris hematoxylin (Sigma). Sections of each cystic kidney were processed in parallel with appropriate controls. TdT-mediated dUTP nick end labeling (TUNEL) staining was performed using the ApopTag Peroxidase InSitu Apoptosis Detection Kit (Chemicon International, Temecula, CA).

The percent of PCNA-positive or TUNEL-positive cells was determined as described by Tao et al. (54, 57). Digital images were generated using a Zeiss Axiostar microscope equipped with a QImaging Micropublisher 3.3 digital camera. Positive staining cells were counted using IP Lab v3.9 (Scanalytics; BD Bioscience, Rockville, MD) by an observer blinded to treatment modality. Noncystic tubules were defined as tubules <50 μm in diameter. At least 15 independent regions per section were randomly selected. To avoid confusion between noncystic tubules and small cysts, as well as potential changes in tubular cells lining large cysts, PCNA-positive tubular cells and TUNEL-positive cells were counted in “medium-sized cysts” of ≈250-μm diameter and expressed as percent of field.

**Biochemical studies.** For calculation of GFR, inulin concentrations were determined by the macro-anthrone method. ERPF was determined by PAH clearance, using colorimetric methodology. Urine protein was measured by precipitation with 3% sulfosalicylic acid (Sigma). Urinary albumin (Exocell, Nephrat II, Philadelphia, PA) and NAG (Roche Diagnostics, Mannheim, Germany) were measured by ELISA.

**Statistical analysis.** Values are reported as means ± SE. Statistical analysis was performed by ANOVA followed by computation of modified t-values according to the method of Bonferroni. Values that were not normally distributed were analyzed by nonparametric methods. Statistical significance was defined as P < 0.05.

**RESULTS**

**Systemic and structural parameters.** Systemic and renal somatic parameters are depicted in Table 1. As in prior studies (28, 51), cystic males receiving vehicle (Cys/Veh) exhibited systemic hypertension compared with noncystic controls. Compared with Cys/Veh rats, values for systolic blood pressure were slightly reduced by Orch and 2-OHE, but not 2-ME. Proteinuria was increased in Cys/Veh rats compared with controls, but reduced to normal levels in rats with Orch, 2-ME, or 2-OHE treatment. Values for urinary albumin excretion were increased in Cys/Veh rats, and significantly reduced by
2-OHE. Values for urinary excretion of NAG, a putative marker of tubular injury in PKD, did not differ among groups. Body weight (BW) gain was numerically lower in Cys/Veh rats, compared with controls, and significantly lower in all treated groups. Left kidney weight (LKW) was massively increased in Cys/Veh rats. LKW was insignificantly reduced by Orch, but significantly reduced by 2-ME, and more so by 2-OHE. The LKW/BW ratio was markedly elevated in Cys/Veh rats, and only modestly attenuated in the Orch group, but significantly limited by both E2 metabolites. 2-OHE was most effective; LKW/BW ratios in Cys/2-OHE rats were significantly lower than those in Cys/Veh and Cys/Orch rats.

Changes in LKW reflected changes in cyst burden, measured as percent of renal cortex (Fig. 1). The percent of cortex occupied by cysts was not significantly reduced by Orch or 2-ME, but it was markedly attenuated by 2-OHE.

Renal function and hemodynamics. Cystic rats exhibited marked reductions in GFR and ERPF, in absolute terms (not shown) and when factored for BW (Fig. 2). Renal function was not significantly improved in cystic rats by Orch or 2-ME. However, in rats receiving 2-OHE, GFR and ERPF were preserved at levels near those in noncystic rats and were significantly improved over those in Cys/Veh rats. Compared with control, values for RVR were significantly increased in Cys/Veh rats. Orch and 2-OHE, but not 2-ME, significantly reduced RVR. Thus, 2-OHE was associated with preservation of both renal structure (as assessed by kidney growth and cyst burden) and renal function.

Mechanisms of 2-OHE protection. Further studies explored the protective mechanisms of 2-OHE. Because 2-ME and Orch were only partially protective, mechanistic studies concentrated on effects of 2-OHE. Three prominent mechanisms of cyst growth include stimulation of RTE proliferation, stimulation of apoptosis, and effects on angiogenesis. Proliferating cells, assessed as the number of cells staining positive for PCNA, were infrequent in noncystic control rats (Fig. 3). In cystic rats, tubules and developing cysts displayed clusters of PCNA-positive cells. In rats receiving 2-OHE, PCNA immunoreactivity was much reduced, to only occasional positive cells. These findings corresponded to renal expression of the
antiproliferative cyclin-dependent kinase inhibitor p21\textsuperscript{Waf1/Cip1} (p21), which was reduced in cystic males, but preserved at near-normal levels in rats treated with 2-OHE (Fig. 4). Apoptosis, assessed as the number of TUNEL-positive cells, was increased in tubules of Cys/Veh rats compared with noncystic animals, and significantly reduced in tubular cells and cyst walls by 2-OHE (Fig. 5).

To explore the role of altered control of angiogenesis, expressions of HIF-1\textalpha and VEGF were measured (Fig. 6). Renal expression of HIF-1\textalpha was upregulated in cystic kidneys, as has been reported (1, 56), but expression was significantly reduced by 2-OHE. VEGF protein expression was lower in cystic kidneys, and further reduced by 2-OHE treatment.

The mTOR has also been implicated in experimental PKD (18, 48, 55, 66). In cystic kidneys, renal expressions of total and p-mTOR were increased, and 2-OHE treatment significantly limited renal mTOR expression (Fig. 7). mTOR is a critical molecule in the initiation of mRNA translation and protein synthesis, and it is functionally linked to a spectrum of upstream and downstream mediators (27). In this context, we evaluated several molecules operating in the control of protein translation (Fig. 7). The serine/threonine kinase Akt, a multifunctional signaling molecule, acts both upstream and downstream of mTOR (44). Serine 473 phosphorylation of Akt kinase was enhanced in cystic rats compared with controls and normalized by 2-OHE. Phosphorylation of S6K and 4E-BP1, substrates for rapamycin-sensitive phosphorylation by mTOR, was also increased in cystic rats and reduced in rats receiving 2-OHE. Enhanced phosphorylation of mTOR, Akt, S6K, and 4E-BP1, indicating activation of this pathway, was accompanied by increased total protein expression of mTOR and Akt in cystic rats, and downregulated by 2-OHE treatment (Fig. 7). These findings suggest that these molecules are regulated on both transcriptional and posttranslational levels in the cystic kidney. Together, these data indicate that modification of certain prominent signaling mediators invoked in the pathogenesis of PKD may be involved in the protective effect of 2-OHE.

**DISCUSSION**

There are currently no approved pharmacologic interventions to slow the progression of clinical PKD. The present data indicate striking preservation of renal structure and function with a novel intervention, 2-OHE, in the Han:SPRD rat model of PKD. The degree of structural protection was comparable with other proposed interventions, including rapamycin (18, 48, 55, 66), tolvaptan (19), and roscovitine (5). Furthermore, while many studies use less precise markers to assess renal function, our studies show a clear benefit in preservation of GFR and ERPF, using precise clearance methods. We also observed partial beneficial effects of other treatments on blood pressure (Orch), proteinuria (Orch, 2-ME), albuminuria (Orch), and LWK (2-ME), as well as a trend toward lower cyst burden and preserved kidney function. However, the protective effects of those interventions were less complete than those observed with 2-OHE.

Both 2-ME and 2-OHE exhibit beneficial effects in vitro. Tofovic et al. reported protection with 2-ME in models of insulin resistance (75) and NOS inhibition-induced renal injury (61), and with 2-OHE in insulin resistance (60), puromycin nephropathy (59), and NOS inhibition-induced injury (61). In the latter model, 2-ME was slightly more effective than 2-OHE. We expected equivalent benefits with 2-ME and 2-OHE, because conversion of 2-OHE to 2-ME in rat plasma is very efficient, 2-OHE is bioequivalent to 2-ME (74), and a robust methylation pathway for conversion is present in the kidney (73). Doses used in the present study were similar to those used in other models (59–61). The stronger effects of 2-OHE are unexplained, considering equivalence in prior studies, although 2-ME has a lesser volume of distribution than 2-OHE in the rat (74), a characteristic that may contribute to the disparate effects. Recently, another group reported failure of 2-ME to affect progression in polycystic rodents (1).

These data also shed light on mechanisms by which 2-OHE limits the progression of PKD. Cystic disease features an imbalance between RTE proliferation and apoptosis (12). Proliferation of RTE (12) and interstitial cells (42) is associated with overexpression of ECM components. Pharmacological inhibition of proliferation limits progression of experimental
PKD (5, 48, 55, 66). The present studies confirm the presence of increased proliferation, and they suggest that inhibition of proliferation may be an important protective mechanism of 2-OHE.

The observed decrease in renal p21 expression is consistent with a proproliferative phenotype in cystic kidneys. p21 inhibits cell proliferation and induces resistance to apoptosis (40, 69)–both desirable in PKD. Deletion of p21 alone is insufficient to produce a cystic phenotype, but loss of p21 in disease states leads to a dedifferentiated proliferative phenotype (29), and impaired resistance to acquired stress insults (38). We (40) and others (3, 47) found decreased expression of p21 in experimental PKD. Polycystin-1 induces p21 via the JAK-STAT pathway (3), and RTE cells stably expressing polycystin-1 (and p21) have reduced rates of proliferation and are resistant to apoptosis (47). Moreover, the mitogenic actions of Akt kinase, which is activated in the cystic kidney, are due in part to its inhibition of p21 (76). Renal p21 expression is reduced in cystic males but not females, suggesting an influence of sex hormones (40). 2-ME (6) and E2 (71) upregulate p21 in vitro, but effects of 2-OHE have not been previously reported. Thus, our data suggest that the protective effects of 2-OHE may relate, at least in part, to induction (or prevention of reduced expression) of p21. The effect of E2 and its derivatives on p21 together with the lack of p21 reduction in female cystic rats raise the question of whether 2-OHE is as effective in females as in males. Our study suggests multiple mechanisms whereby 2-OHE may impact the progression of PKD, some of which are unrelated to p21 expression. Therefore, 2-OHE may also be a promising new treatment for females with PKD.

In PKD, apoptosis is abnormally persistent and can destroy renal parenchyma, allowing cystic epithelial proliferation. Anti-apoptotic interventions limit cystic progression in experimental PKD (12, 54, 57). Our studies confirm the presence of increased apoptosis in cystic kidneys, and the suppressive effect of 2-OHE may be another protective mechanism.

A third mechanism of cyst progression relates to angiogenesis. HIF-1α is a transcription factor implicated in angiogenesis and upregulated in renal ischemia models (21). Our studies confirm the report of Tao et al. (56) that renal HIF-1α expression is increased in this model. PKD is a state of renal vasoconstriction (28), and parenchymal ischemia is believed to contribute to hypertension and loss of renal function. The resulting hypoxic conditions inhibit HIF-1α degradation and lead to its stabilization. In addition, HIF-1α can be induced in an oxygen-independent manner through the Akt-mTOR pathway (16), a likely mechanism in the cystic kidney. Indeed, 2-OHE-induced reduction of HIF-1α expression paralleled suppression of phosphorylation of mTOR and associated signaling molecules. However, a recent report indicates that HIF-1α suppression is not protective in PKD (1), so the importance of this mechanism remains to be determined.

VEGF is an important effector of angiogenesis downstream of HIF-1α and has been implicated in PKD (2, 56, 68). In contrast to some studies, but in agreement with our previous report (51), we found reduced VEGF expression in cystic kidneys. These findings do not necessarily preclude a role of VEGF in cystogenesis, however. Our studies were performed in the late stages of PKD, when lower VEGF expression may be a consequence of progressive interstitial fibrosis resulting in loss of the peritubular capillary network, an important source of renal VEGF (26). In contrast to estrogens, which promote angiogenesis (35), 2-ME and 2-OHE are anti-angiogenic, and this mechanism may have contributed to protection by 2-OHE, particularly at earlier stages of the disease when the renal...
mTOR is at the center of an intricate network of signaling molecules controlling many cell functions relevant to PKD. In addition to promoting cell proliferation and angiogenesis, mTOR is critical in the initiation of mRNA translation and protein synthesis (27). We examined major mediators in this pathway (S6K and 4E-BP1), the former of which also acts as an mTOR kinase controlling serine 2448 phosphorylation of mTOR (22). Cystic rats displayed enhanced phosphorylation of S6K and 4E-BP1, adding support to the notion that mRNA translation and protein synthesis are activated in the cystic kidney.

There is increasing evidence that suppression of mTOR and its signaling network by rapamycin is nephroprotective in diverse models (33), including experimental PKD (18, 48, 49, 55, 66). Potential mechanisms include reduction of RTE proliferation (55), suppression of the downstream signaling molecule S6K (66), and interference with formation of the tuberin-Rheb-mTOR complex by polycystin-1 (48), although protection has been noted in models not associated with Pkd1 mutations (48). There are to our knowledge no published studies directly linking E2 metabolites to mTOR. Our studies present novel data indicating that suppression of Akt, mTOR, and the downstream effectors S6K and 4E-BP1 may be mechanisms of protection with 2-OHE. Importantly, recently published results of clinical trials evaluating rapamycin in PKD (46, 67) have not confirmed promising observations in some experimental studies. Although reasons for disparities between the clinical and experimental studies with rapamycin are not clear, 2-OHE might be a more favorable option to modulate mTOR signaling complex in clinical settings, at least for the lack of immunosuppressive actions.

mTOR and downstream signaling molecules operating in the regulation of mRNA translation are also under control of other signaling systems, including AMP-activated protein kinase (AMPK)(45). When activated, AMPK inhibits mTOR activity. We cannot exclude the possibility that AMPK is altered in the cystic kidney. Indeed, the role of AMPK in mTOR activation and the pathogenesis of PKD have been recently supported by observations in a murine model showing that stimulation of AMPK with metformin ameliorates cyst development (53). In parallel, different lines of evidence indicate that AMPK is one of the effectors of the estrogen receptor (ER). For example, in vascular smooth muscle, Gayard et al. (20) recently demonstrated that pretreatment with an ER antagonist prevented AMPK activation by E2. Of note, 2-OHE, but not 2-ME, activates AMPK in C2C12 myotubes (10), providing a possible mechanism explaining the superior protective effects of 2-OHE compared with 2-ME. AMPK is under control of the tumor suppressor kinase LKB1 (34), which is known to induce G1 cell cycle arrest (58), a mechanism consistent with therapeutic actions in PKD, and the deacetylase SIRT1, which acts as an activator of the LKB1-AMPK cascade (23). Importantly, most recent studies have linked SIRT1 and LKB1 to the ER and identified those molecules as important mediators for ER signaling (13, 36, 72). In addition, activation of the SIRT1-LKB1-AMPK pathway has been implicated in actions of phytoestrogens, such as resveratrol (23).

However, the direct links between the genetic abnormalities specific for the disorder, mTOR, and increased activity of Akt (which is not related to AMPK activity) suggest that mTOR might be activated by multiple mechanisms in PKD. Recent studies with a variety of inhibitors have shown a spectrum of kinases and signaling systems that might be

architecture is still preserved. Interestingly, 2-OHE further decreased VEGF in cystic kidneys, corresponding to the same effect on HIF-1α nuclear expression, suggesting specific actions in the tubular and/or cystic compartments.

mTOR is at the center of an intricate network of signaling molecules controlling many cell functions relevant to PKD. In accord with others (48, 55, 66), we found increased phosphorylation and expression of renal mTOR in cystic rats. Akt kinase, a multifunctional signaling molecule, acts both upstream of mTOR [mediating mTOR phosphorylation via inhibitory phosphorylation of the tuberous sclerosis complex and consequent activation of Rheb GTPase (25)], and downstream of mTOR, being phosphorylated on serine 473 by mTOR in association with the TORC2 complex (45). Similar to mTOR, Akt phosphorylation and expression were increased in cystic rats, as have been reported in this (65) and other (15, 18, 33) PKD models.

In addition to promoting cell proliferation and angiogenesis, mTOR is critical in the initiation of mRNA translation and protein synthesis (27). We examined major mediators in this pathway (S6K and 4E-BP1), the former of which also acts as an mTOR kinase controlling serine 2448 phosphorylation of mTOR (22). Cystic rats displayed enhanced phosphorylation of S6K and 4E-BP1, adding support to the notion that mRNA translation and protein synthesis are activated in the cystic kidney.

There is increasing evidence that suppression of mTOR and its signaling network by rapamycin is nephroprotective in diverse models (33), including experimental PKD (18, 48, 49, 55, 66). Potential mechanisms include reduction of RTE proliferation (55), suppression of the downstream signaling molecule S6K (66), and interference with formation of the tuberin-Rheb-mTOR complex by polycystin-1 (48), although protection has been noted in models not associated with Pkd1 mutations (48). There are to our knowledge no published studies directly linking E2 metabolites to mTOR. Our studies present novel data indicating that suppression of Akt, mTOR, and the downstream effectors S6K and 4E-BP1 may be mechanisms of protection with 2-OHE. Importantly, recently published results of clinical trials evaluating rapamycin in PKD (46, 67) have not confirmed promising observations in some experimental studies. Although reasons for disparities between the clinical and experimental studies with rapamycin are not clear, 2-OHE might be a more favorable option to modulate mTOR signaling complex in clinical settings, at least for the lack of immunosuppressive actions.

mTOR and downstream signaling molecules operating in the regulation of mRNA translation are also under control of other signaling systems, including AMP-activated protein kinase (AMPK)(45). When activated, AMPK inhibits mTOR activity. We cannot exclude the possibility that AMPK is altered in the cystic kidney. Indeed, the role of AMPK in mTOR activation and the pathogenesis of PKD have been recently supported by observations in a murine model showing that stimulation of AMPK with metformin ameliorates cyst development (53). In parallel, different lines of evidence indicate that AMPK is one of the effectors of the estrogen receptor (ER). For example, in vascular smooth muscle, Gayard et al. (20) recently demonstrated that pretreatment with an ER antagonist prevented AMPK activation by E2. Of note, 2-OHE, but not 2-ME, activates AMPK in C2C12 myotubes (10), providing a possible mechanism explaining the superior protective effects of 2-OHE compared with 2-ME. AMPK is under control of the tumor suppressor kinase LKB1 (34), which is known to induce G1 cell cycle arrest (58), a mechanism consistent with therapeutic actions in PKD, and the deacetylase SIRT1, which acts as an activator of the LKB1-AMPK cascade (23). Importantly, most recent studies have linked SIRT1 and LKB1 to the ER and identified those molecules as important mediators for ER signaling (13, 36, 72). In addition, activation of the SIRT1-LKB1-AMPK pathway has been implicated in actions of phytoestrogens, such as resveratrol (23).

However, the direct links between the genetic abnormalities specific for the disorder, mTOR, and increased activity of Akt (which is not related to AMPK activity) suggest that mTOR might be activated by multiple mechanisms in PKD. Recent studies with a variety of inhibitors have shown a spectrum of kinases and signaling systems that might be
involved in the pathogenesis of PKD. These include observations of protective effects of inhibition of Erk MAPKs (39), Raf (4), cyclin-dependent kinases (5), and the EGF receptor (64) as well as nonreceptor tyrosine kinases (52). Therefore, we cannot exclude a contribution of inhibition of other signaling systems by 2-OHE treatment. Studies focusing on the contribution of these pathways to protective effects of 2-OHE in PKD represent possible future research directions in this field.

In summary, 2-OHE is a novel and promising therapeutic agent in PKD. 2-OHE is strikingly effective at limiting cyst growth and preserving renal function in cystic male rats. Protective mechanisms include suppression of proliferation, apoptosis, and angiogenesis. Protection may relate, at least in part, to its actions to preserve p21 levels, and to suppress Akt, mTOR, and associated molecules relevant to control of cell growth, viability, and protein synthesis. Further studies to test the efficacy of 2-OHE in other models, and to verify the safety profile, will help to define the role of this novel therapy. Whether 2-OHE will adversely affect polycystic liver disease, as has been reported with estrogen therapy (7), remains to be determined. Of interest, liver cysts in patients with ADPKD exhibit increased expression of p-Akt, p-mTOR, and p-S6K (41); 2-OHE might be protective in that organ, as well. From the evidence thus far, mTOR inhibitors have not yet proven effective in clinical PKD (46, 67). The present studies suggest that further exploration of novel estrogen metabolites may reveal safe and effective interventions to slow the progression of PKD.

ACKNOWLEDGMENTS

We are grateful to Dr. Kenneth D. Stringer for helpful discussions and intellectual input to this project.

GRANTS

These studies were supported by grants from the National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (DK 078807) and the Polycystic Kidney Research Foundation.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

1. Belibi F, Zafar I, Ravichandran K, Segvic AB, Jani A, Ljubanovic DG, Edelstein CL. Hypoxia-inducible factor-1α (HIF-1α) and autophagy


