20-HETE Mediates Proliferation of Renal Epithelial Cells in Polycystic Kidney Disease

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ABSTRACT
Polycystic kidney diseases are characterized by abnormal proliferation of renal epithelial cells. In this study, the role of 20-hydroxyeicosatetraenoic acid (20-HETE), an endogenous cytochrome P450 metabolite of arachidonic acid with mitogenic properties, was evaluated in cystic renal disease. Daily administration of HET-0016, an inhibitor of 20-HETE synthesis, significantly reduced kidney size by half in the BPK mouse model of autosomal recessive polycystic kidney disease. In addition, compared with untreated BPK mice, this treatment significantly reduced collecting tubule cystic indices and approximately doubled survival. For evaluation of the role of 20-HETE as a mediator of epithelial cell proliferation, principal cells isolated from cystic BPK and noncystic Balb/c mice were genetically modified using lentiviral vectors. Noncystic Balb/c cells overproducing Cyp4a12 exhibited a four- to five-fold increase in cell proliferation compared with control Balb/c cells, and this increase was completely abolished when 20-HETE synthesis was inhibited; therefore, this study suggests that 20-HETE mediates proliferation of epithelial cells in the formation of renal cysts.


Polycystic kidney diseases (PKD) comprise a group of renal genetic disorders characterized by the development and enlargement of fluid-filled cysts and progressive renal failure.1,2 Autosomal dominant PKD (ADPKD) has an estimated prevalence of 1:400 to 1:1000 and is responsible for approximately 5 to 10% of end-stage renal failure requiring renal replacement therapy, including dialysis and/or whole-kidney transplantation.3,4 Approximately 25% of patients with autosomal recessive PKD (ARPKD), which has an incidence of 1:10,000 to 1:40,000, die in the newborn period, with survivors generally progressing to ESRD during childhood or adolescence.5 The ESRD care of patients with PKD in the United States alone will cost more than $2 billion in 2008. Currently, there is no disease-specific therapy for PKD. Given the similarities in the pathophysiology of cyst formation and progressive enlargement in both ADPKD and ARPKD, it seems likely that an effective therapy developed for one genetic form of this disease would have overlap to be effective for the other form.6

An early and consistent pathophysiologic feature of human PKD and its experimental animal models is increased epithelial cell proliferation.6–9 In the past decade, a number of specific therapies targeting the proliferative phenotype of PKD have attenuated the severity of the cystic phenotype, ameliorated decline in renal function, and enhanced survival in a number of animal models of PKD.3,10 Recent evidence has implicated 20-hydroxyeicosatetraenoic acid (20-HETE), which is formed by the ω-hydroxylation of arachi-
stimulate the synthesis and release of 20-HETE and increase epinephrine, angiotensin II (AngII), and EGF have been shown to include those present within the kidney. In these studies, nornepinephrine, angiotensin II (AngII), and EGF were shown to stimulate the synthesis and release of 20-HETE and increase thymidine incorporation in vascular smooth muscle cells and proximal tubular epithelial cells in the kidney. These effects were shown to be blocked by selective inhibitors of 20-HETE synthesis. Moreover, these findings were consistent with recent studies that 20-HETE synthesis inhibitors N-hydroxy-N′-(4-butyl-2-methylphenol) formamidine (HET-0016) and DDMS inhibited the proliferation of gliomas in vitro and growth of 9L tumors in vivo. To date, there remains a paucity of information regarding the role of AA metabolites in the cellular proliferation of renal epithelium in PKD. For these reasons, this study was designed to determine the role of eicosanoids, in particular 20-HETE, in mediating the abnormal epithelial cell proliferation of PKD in vitro and in vivo using the Balb/c polycystic kidney (BPK) mouse, which is a well-characterized model of ARPKD.

RESULTS

Effects of Long-Term Administration of 20-HETE on Cystogenesis in BPK Mice

To investigate the effect of cytochrome P450 4A and 4F blockade in vivo, we studied the well-characterized BPK murine model of ARPKD, because of its consistent cystic phenocopy with humans as well as its short life span (<28 d), allowing for rapid screening of therapeutic drugs. We administered HET-0016 (10 mg/kg per d intraperitoneally), a specific 20-HETE synthesis inhibitor, on a daily basis from postnatal day 7 to postnatal day 20. On day 21, the mice were killed and the kidneys were analyzed. In Figure 1, there was a marked increase in the size of vehicle-treated cystic BPK kidneys (Figure 1B) compared with noncystic Balb/c kidneys (Figure 1A), and treatment with HET-0016 resulted in a dramatic decrease in kidney size (Figure 1C). As shown in Table 1, the whole kidney weight/body weight ratio averaged 1.36 ± 0.05% (n = 5) and 1.34 ± 0.07% (n = 5) in noncystic Balb/c mice untreated or treated with HET-0016, respectively, and both groups were significantly lower (P < 0.001) than the untreated cystic BPK mice (20.0 ± 0.4%; n = 7). Long-term blockade of 20-HETE synthesis significantly lowered the kidney weight/body weight ratio to 10.2 ± 0.9% (P < 0.001; n = 7) in the BPK mice, which was approximately 50% lower than that of the untreated BPK mice. More importantly, we were able to demonstrate that the survival of the cystic BPK mice could be significantly (P = 0.011E extended on average for an additional 20 d (43.3 ±

Table 1. Biological parameters of noncystic Balb/c (+/+) and BPK (--/--) mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Balb/c (+/+)</th>
<th>BPK (--/--)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>HET-0016</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>10.50 ± 0.30</td>
<td>10.40 ± 0.20</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>KW/BW (%)</td>
<td>1.36 ± 0.05</td>
<td>1.34 ± 0.07</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>PN-21 CT CI</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>18.30 ± 0.30</td>
<td>18.30 ± 0.70</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.23 ± 0.03</td>
<td>0.20 ± 0.00</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 4)</td>
</tr>
</tbody>
</table>

*Body weight and kidney weight were measured in postnatal day 21 Balb/c and BPK mice to calculate the ratio of kidney weight/body weight (KW/BW). Kidneys were harvested and sectioned to evaluate the collecting tubule cystic indices (PN-21 CT CI) from Balb/c and BPK mice treated with or without HET-0016, and blood was collected to examine the BUN and creatinine.

bP < 0.001 untreated BPK mice compared with all of the other mouse groups.
1.5 d of survival) compared with untreated BPK mice (23.5 ± 0.7 d of survival) as shown in Figure 2.

Histologic examination of the kidneys from the vehicle- and HET-0016–treated BPK mice demonstrated marked reductions in the size and number of renal cysts (Figure 3). In the vehicletreated BPK mice, the cysts were increased in both number and size (Figure 3A) compared with the HET-0016–treated mice (Figure 3B). For untreated cystic BPK mice, the collecting tubule cystic index as shown in Table 1 was 4.4 ± 0.3 (n = 5), and there was a significant reduction (P < 0.001) in the collecting tubule cystic index after treatment with HET-0016 to 2.2 ± 0.3 (n = 5). No cysts were found in noncystic Balb/c mice treated or untreated with HET-0016 (n = 5 for each group).

Renal function was determined by measurement of blood urea nitrogen (BUN) and creatinine from the untreated and treated noncystic Balb/c and cystic BPK mice (Table 1). The plasma BUN in untreated Balb/c mice (18.3 ± 0.3 mg/dl; n = 3) was significantly lower (P < 0.001) than in its untreated BPK mice counterparts (100.3 ± 7.1 mg/dl; n = 4). In the presence of HET-0016 (10 mg/kg per d), no significant effect was detected comparing treated and untreated Balb/c mice; however, there was a significant reduction in the plasma BUN to 34.4 ± 2.6 mg/dl (n = 5) compared with the untreated BPK mice, indicating that renal function was more normal in the presence of a 20-HETE synthesis inhibitor. Similar trends were also found in the serum analyses for creatinine (Table 1). These results strongly suggest that 20-HETE inhibition can protect renal function and prolong animal survival.

Effect of 20-HETE Inhibition on 20-HETE Production in Noncystic Balb/c and Cystic BPK Mouse Kidneys

For further examination of the biological role of 20-HETE in the epithelial cell proliferation, 7- to 11-d-old BPK mice were treated with an intraperitoneal injection of vehicle or HET-0016 (10 mg/kg per d) 90 to 120 min before being killed, at which point the kidneys were removed for measurement of
20-HETE production. As shown in Figure 4A, the production of 20-HETE was significantly higher ($P < 0.05$) in the renal BPK microsomes (1.06 ± 0.56 pmol/min per mg protein; $n = 6$) compared with the levels detected in the noncystic Balb/c mouse microsomes (0.13 ± 0.01 pmol/min per mg protein; $n = 11$). Administration of HET-0016 (10 mg/kg per d) before the killing of the mice demonstrated that there was selective inhibition in the formation of 20-HETE by >92% in the BPK mice (0.08 ± 0.03 pmol/min per mg protein; $n = 5$) with minimal affect of HET-0016 on other metabolites of AA breakdown in the kidneys (Figure 4B). It is important to note that no significant effect ($P > 0.05$) on 20-HETE production was determined after HET-0016 treatment in noncystic Balb/c mice (0.06 ± 0.02 pmol/min per mg protein; $n = 4$).

**Figure 4.** Production of 20-HETE in cystic and noncystic mouse kidneys. HET-0016, a specific inhibitor of cytochrome P450 4A and 4F isoforms, was administered at a dosage of 10 mg/kg per d intraperitoneally to noncystic Balb/c and cystic BPK mice 90–120 min before being killed between postnatal days 7 and 11. Kidneys were harvested, and microsomal protein was isolated for 20-HETE production assay in vitro. Lipids were extracted using ethyl acetate and water, and the final samples were analyzed to determine the level of 20-HETE production as well as other metabolites of AA using liquid chromatography–quadruple mass spectroscopy (LC-MS). (A) Demonstrated the 20-HETE production with vehicle (VEH) or in the presence of HET-0016 (HET; 10 mg/kg) between cystic (BPK) and noncystic (Balb/c) mice. **$P < 0.05$ significance between the BPK (VEH) versus Balb/c (VEH). (B) Demonstrated the eicosanoid profile of other AA metabolites in the presence and absence of HET-0016 (10 mg/kg) in the cystic (BPK) and noncystic (Balb/c) mice; $n = 4$ to 11 mice/group.

Molecular Determination of the Cytochrome P450 4A Isoforms in the Conditionally Immortalized Renal Epithelial Cells Isolated from Noncystic Balb/c and Cystic BPK Mouse Kidneys

To determine which CYP isoforms are expressed and may contribute to the formation of 20-HETE in cystic epithelial cells, we designed specific PCR primers to determine whether the cytochrome P450 4A and 4F isoforms would be differentially expressed in cystic BPK versus noncystic Balb/c epithelial cells (Table 2). The RT-PCR analysis was performed in conditionally immortalized renal epithelial cells isolated from the intercrossed BPK × ImmortoMouse as described by Sweeney et al.19 From our analysis, we found that there were low-level steady-state levels of Cyp4a10 mRNA in the noncystic Balb/c cells (158.2 ± 35.5 copies/μg RNA; mean ± SEM; $n = 4$) with a slight, nonsignificant increase ($P > 0.05$) in the cystic BPK cells (595.3 ± 252.5 copies/μg RNA; $n = 5$). For the Cyp4a12 mRNA, the steady-state levels in Balb/c cells were 43,664 ± 32,171 copies/μg RNA ($n = 4$), which was significantly higher ($P < 0.001$) than the Cyp4a10 mRNA levels in either BPK or Balb/C cells ($n = 4$ to 5). Interestingly, the Cyp4a12 mRNA in the cystic BPK cells was approximately 10-fold higher ($P < 0.001$) at 369,053 ± 40,177 copies/μg RNA ($n = 5$). No detectable copies of Cyp4a14 mRNA was detected in either the BPK or Balb/c cells ($n = 4$/cell line). From our reverse transcription–PCR results, the increased steady-state levels of Cyp4a12 isoform indicated a potential biologic role in mediating epithelial cell proliferation.

**Genetic Modification of Conditionally Immortalized Renal Epithelial Cells Using VSV-G Pseudotyped Lentiviral Vectors**

Full-length Cyp4a10 and Cyp4a12 cDNA driven by the human ubiquitin C promoter were cloned into modified lentivector transfer plasmids previously developed by Park and Kay20 (Figure 5). Lentiviral vectors were generated and serially transduced into nonpermissive Balb/c epithelial cells at an approximate multiplicity of infection (MOI) of 40. All of the cells transduced by the lentiviral vectors expressed the EGFP transgene as determined by FACS analysis (Figure 6A), and no spu-

**Table 2.** mRNA levels of cytochrome P450 4A isoforms

<table>
<thead>
<tr>
<th>Gene</th>
<th>Noncystic Balb/c Cells (copies/μg RNA)</th>
<th>Cystic BPK Cells (copies/μg RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp4a10</td>
<td>158.2 ± 35.5 ($n = 4$)</td>
<td>595.3 ± 252.5 ($n = 5$)</td>
</tr>
<tr>
<td>Cyp4a12</td>
<td>43,664.0 ± 32,171.0 ($n = 4$)</td>
<td>369,053.0 ± 40,177.0 ($n = 5$)</td>
</tr>
<tr>
<td>Cyp4a14</td>
<td>UND ($n = 4$)</td>
<td>UND ($n = 4$)</td>
</tr>
</tbody>
</table>

*This table documents real-time RT-PCR results demonstrating the steady-state levels of CYP4A isoforms in the cystic BPK and noncystic Balb/c cells. The cells were harvested 10 d after culturing under nonpermissive conditions, and total RNA was extracted using TRIzol reagent. RT-PCR was performed using specific primers against the murine Cyp4a10, Cyp4a12, and Cyp4a14 genetic sequence. UND, undetectable.

$^b$P < 0.001 difference in the levels of Cyp4a12 mRNA between cystic BPK and noncystic Balb/c cells.
rious fluorescence was noted in any of the vehicle-treated noncystic Balb/c cells or both of the lentiviral vector–transduced Balb/c cells expressing both of the CYP4A isoforms.

The functional activity of the cytochrome P450 4A isoforms was determined using liquid chromatography coupled to triple quadrupole mass spectrometry following 20-HETE production assay (Figure 6B). This showed that the 20-HETE production was significantly higher (*P < 0.001) in the Cyp4a12 expressing cells (n = 3) compared with the untreated EGFP-expressing Balb/c cells (n = 4). Similar increases in 20-HETE production were observed using Cyp4a10 transduced Balb/c cells, and this was also correlated to increased protein detection of Cyp4a10 by Western blot analysis (data not shown).

In addition, the lentiviral vector–mediated overexpression of Cyp4a12 to synthesize 20-HETE could be markedly inhibited by 70% (n = 2) after administration of HET-0016 at a dosage of 1 μM during the 20-HETE production assay. No significant differences were noted in the production of any of the other HETE, dihydroxyeicosatetraenoic acid, epoxyeicosatrienoic acid, and prostaglandins using the lentivector–manipulated noncystic Balb/c cells expressing EGFP or Cyp4a12. *P < 0.001 difference between Cyp4a12 versus noncystic Balb/c cells treated with vehicle (VEH) or HET-0016 (HET). VEH, vehicle (PBS); EGFP, Balb/c cells transduced with lentiviral vectors expressing EGFP (GFP); Cyp4a10 and Cyp4a12, cytochrome P450 4a10 and 4a10 isoforms; HET, HET-0016 (10 μM).

**Effect of 20-HETE Overproduction on Cell Proliferation in Noncystic Balb/c and Cystic BPK Renal Epithelial Cells**

The cells in this experiment were transduced using the lentiviral vectors in the presence of γ-IFN (i.e., permissive state) and allowed to expand. Once the cells were expanded, the transduced cells were grown in the absence of γ-IFN (i.e., nonpermissive state) for 10 d. The cells were harvested and counted to initiate the proliferation experiment.

Under nonpermissive conditions, cystic BPK cells were significantly increased (P < 0.001) in cell number to 256,700 ± 17,136 (n = 4) by day 3 relative to the noncystic Balb/c cells (132,892 ± 11,925; n = 6) at the end of the 5-d experiment (Figure 7A). In the presence of a 20-HETE synthesis inhibitor, HET-0016 (10 μM), the number of cystic BPK cells was significantly reduced (P < 0.001) to 40,744 ± 4322 (n = 4) by day 2 compared with the vehicle-treated BPK cells. No marked difference in the Balb/c epithelial cells treated with HET-0016 (99,600 ± 8058; n = 4) was noted at the end of the 5-d period compared with the vehicle-treated Balb/c cells (132,892 ± 11,925; n = 6). Similar to the cystic BPK cells, there was a tendency for the cells to be decreased in number after treatment with HET-0016 (10 μM) relative to the vehicle-treated cells, indicating that an alternative eicosanoid may also play a role in their proliferation.

**Figure 5.** Schematic of lentiviral vector transfer plasmid. The lentiviral vector transfer plasmid was cloned with the human ubiquitin (Ub) promoter driving the expression of the full-length murine cytochrome P450 4a10 (Cyp4a10) and 4a12 (Cyp4a12) cDNA. RRE, rev-responsive element; W, woodchuck postregulatory element; SD, splice donor; SA, splice acceptor; ψ, packaging signal; 3’ SIN LTR, 3’ self-inactivating long-terminal repeat; gray box, central polyuridine tract sequence.

**Figure 6.** Transduction efficiency and transgene expression and function after genetic manipulation using lentiviral vectors on conditionally immortalized murine renal epithelial cells. (A) Graphic analysis of the transduction efficiency of noncystic Balb/c renal epithelial cells treated with vehicle (PBS) or lentiviral vectors expressing EGFP, Cyp4a10, or Cyp4a12. (B and C) Functional analysis of 20-HETE production (B) and other HETE, dihydroxyeicosatetraenoic acid, epoxyeicosatrienoic acid, and prostaglandins (C) using the lentiviral vector–manipulated noncystic Balb/c cells expressing EGFP or Cyp4a12. *P < 0.001 difference between Cyp4a12 versus noncystic Balb/c cells treated with vehicle (VEH) or HET-0016 (HET). VEH, vehicle (PBS); EGFP, Balb/c cells transduced with lentiviral vectors expressing EGFP (GFP); Cyp4a10 and Cyp4a12, cytochrome P450 4a10 and 4a10 isoforms; HET, HET-0016 (10 μM).
role in the proliferative pathway of noncystic Balb/c and cystic BPK cells.

The decreased total number of cystic BPK cells in the presence of HET-0016 was consistent with the 5-bromo-2′-deoxyuridine (BrdU) incorporation experiment (Figure 8), which provides an index of DNA synthesis. BPK epithelial cells were incubated with vehicle (Figure 8A) or HET-0016 (10 and 20 μM; Figure 8, B and C) in the presence of BrdU (30 μM), and after 36 h cells were fixed for immunohistochemistry to measure BrdU incorporation. A total of 500 total BPK cells per plate were counted (Figure 8D), and there was a significant reduction (P < 0.001) in the number of BrdU-positive cells [52 and 38 BrdU(+) cells] compared to the vehicle-treated BPK cells [118 BrdU(+) cells]. Blockade with an alternate inhibitor of 20-HETE synthesis, DDMS, resulted in a similar decrease in the number of BrdU-positive cystic BPK cells to 63 (n = 2) compared with vehicle treatment. Overall, these in vitro experiments demonstrate that 20-HETE mediates the proliferative activity of the cystic BPK cells.

The total number of noncystic Balb/c cells was significantly increased (P < 0.001) after transduction with either Cyp4a10 (347,075 ± 19,635; n = 4) or Cyp4a12 (285,792 ± 21,892; n = 6) compared to the EGFP-transduced Balb/C cells (68,767 ± 4124; n = 6) by day 3 of the experiment (Figure 7B). This demonstrates that the noncystic Balb/c cells could be modified to simulate the proliferative phenotype of the cystic BPK cells by overexpression of Cyp4a10 or Cyp4a12. Moreover, blockade in the production of 20-HETE using HET-0016 (10 μM) dramatically reduced the number of Cyp4a12-expressing Balb/c cells (49,700 ± 5085; n = 3). There was no significant difference (P > 0.05) between Balb/c cells treated with vehicle or the EGFP-expressing lentiviral vectors.
Chronic Inhibition of 20-HETE Synthesis Affects the Activation of the EGF Receptor

Western blot analysis was performed using kidney homogenates harvested from BPK mice at postnatal day 21 in the presence or absence of long-term HET-0016 (10 mg/kg per d) administration. Figure 9A showed EGF receptor (EGFR) phosphorylation at tyrosine residue 1086 (Tyr1086) in BPK mouse kidneys, and a significant 35% reduction was detected (P < 0.001) after HET-0016 treatment (0.63 ± 0.02; n = 3 samples) compared with untreated BPK mouse kidneys (0.97 ± 0.02; n = 3 samples) by densitometry of the bands (Figure 9B). No difference was detected in the loading of the protein samples as determined by the β-actin levels.

DISCUSSION

Renal tubular epithelial cell proliferation is a central pathophysiologic characteristic of renal cyst formation and progressive enlargement in PKD. A number of mediators of cell proliferation have been identified in cells from patients with PKD as well as numerous animal models of PKD; however, the integration and hierarchy of different specific proliferative pathways in mediating disease progression in human ADPKD and ARPKD remain unknown. Such data are important in therapeutic development of maximally effective and minimally toxic disease-specific therapies in PKD.

Recent evidence demonstrates that cytochrome P450 metabolites of AA, including 20-HETE, are endogenously produced in the kidney and can be upregulated during tissue injury. There is strong evidence that 20-HETE functions as a second messenger in signal transduction pathways to modulate vascular tone, sodium excretion, cell proliferation, and angiogenesis. Because of the multifactorial effects of 20-HETE in the kidney, the mechanism by which 20-HETE may be involved in increasing the kidney size and cyst number remains to be elucidated. One possibility is that 20-HETE may pathologically alter fluid flux through the epithelial cells, resulting in increased retention of cyst fluid, and blockade of 20-HETE production promoted a shift in the fluid dynamics to minimize cyst volume. Alternatively, 20-HETE may be involved in mediating epithelial cell proliferation and cyst formation through a mechanism that is yet to be determined.

In particular, there is evidence of significant interactions of such metabolites with the EGFR pathway, which we have consistently implicated as a critical mediator of disease progression in PKD; therefore, we examined the role of such metabolites, particularly 20-HETE, as an effector of epithelial cell proliferation in PKD. In the mouse, 20-HETE is produced by members of the CYP4A subfamily, including Cyp4a10, Cyp4a12, and Cyp4a14. These CYP4A isoforms are known to catalyze a ω-hydroxylation reaction of AA to produce 20-HETE, and alterations in 20-HETE production have been found to be associated with hypertension, cirrhosis, and endothelial dysfunction.

Several reports have linked the 20-HETE-mediated release of AA and its subsequent proliferative response through an interaction with the EGFR using both normally quiescent and actively proliferating oncogenic cells. The interaction of the EGFR with 20-HETE may play an important role in PKD as a result of the well-established effect of EGFR activation as a critical mediator of cyst formation and progressive enlargement of the kidney in human PKD and all animal models of PKD studied to date.

Recent work showed that 20-HETE has been found to promote angiogenesis in vivo in a growth factor–dependent manner, and blockade of the vascular endothelial growth factor receptor abolished the 20-HETE mediated effects. Chen et al. found that WIT003, a 20-HETE agonist, could induce mitogenesis in endothelial cells in vitro and angiogenesis in the rat cornea in vivo. Interestingly, inhibition of 20-HETE synthesis blocked the angiogenic effects of various growth factors, including basic fibroblast and EGF, by 80 to 90% in vivo. Other studies using norepinephrine, AngII, and EGF have been shown to stimulate the synthesis and release of 20-HETE and promote increased thymidine incorporation in vascular smooth muscle cells and proximal tubular epithelial cells in the kidney through a Ras-dependent and -independent activation of the mitogen-activated protein kinase (MAPK) pathway.

More recent studies by Guo et al. demonstrated an interaction between the EGFR, the MAPK pathway, and 20-HETE. Blockade of 20-HETE synthesis significantly reduced the mitotic index of proliferating cancer cells, which
was associated with a concomitant reduction in the phosphorylated (activated) form of the EGFR as well as the downstream signaling pathways involving p42/p44 MAPK in both the human U251 glioma and rat 9L gliosarcoma cells. It is important to note that our study demonstrated a significant reduction in the activation (phosphorylation) of the EGFR (Tyr1086) in the presence of HET-0016 in cystic BPK epithelial cells in vitro (data not shown) and in mouse kidneys in vivo (Figure 9). The decreased activity of the EGFR in these studies may be involved in attenuating the proliferative response of the epithelial cells even in the presence of pro-cystic hormonal stimulation by AngII, endothelin, vasopressin, or other growth factors. There is recent evidence that the MAPK pathway can activate cytosolic phospholipase A2 to release AA in vascular smooth muscle cells. It is possible that the increased release of AA leads to higher levels of 20-HETE, resulting in accelerated cell proliferation through the MAPK and/or alternate pathways that have yet to be determined. Another possibility is the production and release of EGFR ligands by the cystic cells through a 20-HETE–mediated pathway, which ultimately results in the activation of the downstream signaling cascades. Further work is needed for better understanding of how altered CYP4A expression can lead to elevated levels of 20-HETE in cystic animals, and emerging new data linking intracellular signaling cascades involved in cell proliferation between cancer and PKD cells may help to further elucidate the mechanism of 20-HETE–mediated hyperplasia in PKD.

This is the first study to demonstrate that 20-HETE is a novel mediator of epithelial cell proliferation in the BPK model of PKD. Our findings demonstrated that blockade of the cytochrome P450 4A and 4F enzymes, which synthesize 20-HETE, using HET-0016 resulted in a marked reduction in kidney mass, as well as quantitative morphometric indices of cystic disease in the BPK mouse, a well-characterized model of ARPKD. Moreover, we found that there was an increase in the renal epithelial cell number in the lentiviral vector–modified normal renal epithelial cells overexpressing CYP4A isoforms, specifically Cyp4a10 and Cyp4a12, which seemed to simulate the enhanced cell proliferative ability of cystic BPK cells. Our new findings suggest that 20-HETE may be a vital molecule in the activation of cell proliferation in PKD; however, further studies regarding the safety profile after long-term administration of HET-0016 or other similarly acting drugs in PKD will need to be examined so that the development of inhibitors to this pathway may result in a new therapeutic target in the treatment of PKD.

**CONCISE METHODS**

**Chemicals and Reagents**

HET-0016 was purchased from BIOMOL International, LP (Plymouth Meeting, PA).

**BPK Mice**

This study was conducted using "immortalized" BPK mice as previously described by Sweeney et al. In brief, BPK female heterozygotes (bpk+/−) were bred with H-2Kb-ts-A58 transgenic males. Compound heterozygotes (bpk+/−;H-2Kb-ts-A58+/−) were identified by PCR and backcrossing to produce cystic offspring. Compound heterozygotes were mated to generate cystic (bpk−/−;H-2Kb-ts-A58+/−) and noncystic (bpk+/−;H-2Kb-ts-A58+/−) offsprings carrying at least one copy of the Immorto transgene (bpk−/−;H-2Kb-ts-A58+/−). All of the mice were genotyped for the H-2Kb-ts-A58 transgene by PCR analysis of DNA extracts from tail sections as described previously. In some mice, we continued the administration of HET-0016 to examine the survivability of the mice in the presence of long-term inhibition of 20-HETE synthesis.

**Serum Marker Measurement**

Blood analysis of creatinine and BUN, which are indices of renal function, were measured by the diagnostic laboratories at Marshfield Clinic (Marshfield, WI).

**Immunohistology of Cystic BPK Kidneys with and without 20-HETE Inhibition**

Kidney and liver tissues were harvested for qualitative analysis as described previously at postnatal day 21. Briefly, kidney and liver were fixed in 4.0% paraformaldehyde in phosphate buffer (pH 7.4) for 30 min at 4°C. Tissues were then washed, dehydrated through a graded series of acetone, and fixed in Immunobead embedding medium (Polysciences, Warrington, PA). Sections were cut at 4 μm, mounted on glass slides, and stained with hematoxylin or lectins staining the proximal tubule (Lotus tetragonolobus agglutinin) and collecting tubule (Dolichos biflorus agglutinin). Segmental neprhon cyst localization was characterized by light microscopy, and cystic lesions were quantified by a morphometric index as described previously.

**Protein Isolation and Western Blot Analysis of Phospho-EGFR**

Mouse kidneys from BPK mice with and without HET-0016 treatment were homogenized in × radioimmunoprecipitation buffer in the presence of phosphatase and protease inhibitors. Differential centrifugation was performed to harvest the homogenate and aliquotted for protein concentration measurement. Standard immunoblotting techniques were performed as described previously, and membranes were incubated with the primary antibody targeted to the phospho-EGFR (Tyr1086) at a dilution of 1:1000 (Cell Signal, Danvers, MA) overnight and then washed with PBS-T. Subsequently, the membranes were incubated with a secondary anti-rabbit IgG coupled to horseradish peroxidase for 60 min. The membranes were washed and then placed into chemiluminescent solution (Amersham, Piscataway, NJ) before placement onto film for detection of the bands. β-Actin was used as a loading control for the membranes. Band intensity was calculated using National Institutes of Health Image software.
Construction of CYP4A-Expressing Lentiviral Vector Transfer Plasmid
Murine full-length Cyp4a10 (cDNA clone MGC:58977) and Cyp4a12 (cDNA clone MGC:25972) cDNA clones were purchased from ATCC (Manassas, VA). The backbone lentiviral vector transfer plasmid used in these studies was previously described.24 In brief, the lentiviral vector transfer plasmid contained debilitated 3’ long-terminal repeats, a small 118-bp segment from the pol gene known as the central polypurine tract sequence and the woodchuck postregulatory element as shown in Figure 5. For the cloning of the Cyp4a10-expressing transfer plasmid, pHRI(+)-cUb.GFP.R(−)W(+) was double digested with KpnI and XbaI to remove the GFP cDNA and was replaced with the XbaI/KpnI fragment containing the Cyp4a10 cDNA. The final construct was named pHRI(+)-cUb.Cyp4a10.R(−)W(+). For the Cyp4a12-expressing transfer plasmid, an additional XbaI/XbaI fragment containing the 3’ end of the Cyp4a12 cDNA was cloned into the XbaI-digested pHRI(+)-cUb.Cyp4a12(short).R(−)W(+) plasmid to make the final construct, pHRI(+)-cUb.Cyp4a12.R(−)W(+).

Lentiviral Vector Production
Modified lentiviral vectors were produced by using transient triple-plasmid transfection of 293T cells as described previously.58–61 The lentiviral vectors were produced by using the following amounts of plasmid DNA: 10 μg of transfer plasmid, 6.5 μg of packaging plasmid, and 3.5 μg of envelope plasmid. Conditioned media were collected after 48 h, filtered, and frozen at −80°C. Single-channel FACS analysis (Becton Dickinson, Franklin Lakes, NJ) was performed on EGFP-expressing lentiviral vector transduced cells and analyzed with the CellQuest program (Version 3.1f; Becton Dickinson) to determine lentiviral vector titer. 

Noncystic Balb/c (+/+) and Cystic BPK (−/−) Renal Epithelial Cell Isolations
The renal epithelial cell isolation was performed on postnatal day 14 in noncystic Balb/c (+/+) and cystic BPK (−/−) mice as described previously.19,62 The renal epithelial cells were maintained in a serum-free defined medium consisting of a 1:1 mixture of DMEM and Ham’s F-12 medium, supplemented with insulin (8.3 × 10−7 M), prostaglandin E1 (7.1 × 10−8 M), selenium (6.8 × 10−7 M), transferrin (6.2 × 10−8 M), triiodothyronine (2 × 10−9 M), dexamethasone (5.09 × 10−8 M), and recombinant γ-IFN (10 U/ml; Invitrogen Corp., Carlsbad, CA) at 33°C (permissive conditions). The cells were serially transduced with VSV-G pseudotyped lentiviral vectors in the presence of polybrene (8 μg/ml) on a daily basis as the cells were expanded to determine lentiviral vector transduction efficiency by FACS analysis.

In Vitro Experiments Using the Conditionally Immortalized Cell Lines
For the cell counting experiments, the noncystic Balb/c and cystic BPK renal epithelial cells were seeded in six-well dishes and changed into medium lacking γ-IFN at 37°C (nonpermissive conditions) for at least 6 additional days before assessment of any phenotypic analyses to ensure the loss of the T antigen.19 Cells were harvested by tryptic digestion and counted by light microscopy using a hemocytometer.

For determination of the level of proliferation in the cystic BPK cells, chemically dissimilar inhibitors of cytochrome P450 4A (CYP4A) and 4F (CYP4F) inhibitors, specifically HET-0016 (10 and 20 μM) and DDM5 (10 μM), were incubated for 36 h with the BPK cells. Three hours before the harvesting of the cells, BrdU at a concentration of 30 μM was added to determine the level of cell-cycle inhibition. The cells were subsequently methanol fixed, and BrdU-positive cells were identified by immunohistology with biotinylated monoclonal anti-BrdU antibody (Zymed, South San Francisco, CA). Data were expressed as the percentage of BrdU-labeled cells per 500 counted cells.

Reverse Transcription Real-Time Quantitative PCR for Cyp4a10 and Cyp4a12
Total RNA was extracted from the cystic BPK and noncystic Balb/c cells using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). The total RNA (2 μg) was DNase-treated with 1 U of RNase-free DNase (Promega, Madison, WI) for 30 min, and the RNA was reverse-transcribed using oligo-dT primer and SuperScript III RTase (Invitrogen Corp.) for 60 min at 42°C. After cDNA synthesis, the RT products were heated to 85°C for 10 min and immediately placed on ice. All primers for PCR were purchased from Integrated DNA Technologies (Coralville, IA) using gene-specific primers for Cyp4a10, Cyp4a12, and Cyp4a14 as follows: Cyp4a10 sense 5’-GACAAGGACCTAGTGCTGAGG-3’ and antisense 5’-CTCATAGAAATTTGTTCGCCA-3’; Cyp4a12 sense 5’-TGAGTCCTATGAAAGATGTC-3’ and antisense 5’-CTGGGAGCCGAGCAGAGGG-3’ and Cyp4a14 sense 5’-CTCAAAGGATCTTGAGTT-3’ and antisense 5’-ATCATAAGGAGACTCGTATA-3’. Real-time quantitative PCR was performed using Stratagene 3000XP real-time PCR machine and SYBR Green reagents. The PCR reaction mixture contained 1× SYBR Green PCR master mix, 1 U Vent DNA polymerase (NEB), 50 nM forward and reverse primers, and 100 to 250 ng of cDNA in a total reaction volume of 20 μl. Each reaction was performed at the following conditions: 95°C for 10 min, 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s for a total of 40 cycles. Quantitative numbers for CYP4A mRNA expression were calculated by comparing the PCR production against a standard curve, which was generated using diluting amounts of plasmid containing the full-length murine CYP4A cDNAs. The values were expressed as mean copies of mRNA ± SEM.

Liquid Chromatography–Triple Quadrupole Mass Spectrometry for 20-HETE Detection
Microsomes were isolated from the cystic BPK and noncystic Balb/c mouse kidneys as described previously by Ito et al.52 For the BPK and Balb/c cells, protein lysates were harvested by homogenization and subsequent sonication of the cell pellets. For the 20-HETE production
Statistical Analysis

The results are calculated as means ± SEM. The significance of differences between groups was examined by either an unpaired t test or a one-way ANOVA with Prism 4.0 software (GraphPad Software, San Diego, CA) followed by a Newman-Keuls post hoc test. Two-way ANOVA with a Bonferroni post hoc test was performed for the cell culture proliferation assay comparing the differences in the groups for time and drug or vector treatment. For the survival group comparison between untreated and treated BPK mice with HET-0016, the non-parametric Mann-Whitney U test was performed. The level of statistical significance was set at P < 0.05.

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DISCLOSURES

None.

REFERENCES


