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.5

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-
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,18 because of its consistent cystic phenocopy with humans as well as its short life span (<28 d), allowing for rapid screening of therapeutic drugs.18 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 ± 0.03% (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 = 0.0116) 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>HET-0016</th>
<th>BPK(−/−)</th>
<th>HET-0016</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>10.50 ± 0.30</td>
<td>10.40 ± 0.20</td>
<td>10.50 ± 0.30</td>
<td>10.40 ± 0.30</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>2.10 ± 0.08</td>
<td>1.06 ± 0.11</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 7)b</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>KW/BW (%)</td>
<td>1.36 ± 0.05</td>
<td>1.34 ± 0.07</td>
<td>20.00 ± 0.40</td>
<td>10.20 ± 0.90</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 7)b</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>PN-21 CT CI</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>4.40 ± 0.30</td>
<td>2.20 ± 0.30</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 7)b</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>18.30 ± 0.30</td>
<td>18.30 ± 0.70</td>
<td>100.30 ± 7.10</td>
<td>34.40 ± 2.60</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 5)</td>
<td>(n = 4)b</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.23 ± 0.03</td>
<td>0.20 ± 0.00</td>
<td>0.68 ± 0.04</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 4)b</td>
<td>(n = 5)</td>
</tr>
</tbody>
</table>

*aBody 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 vehicle-treated 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.

Figure 2. Effect of 20-HETE synthesis inhibition on survival time in the cystic BPK mouse. Long-term daily administration of HET-0016 (10 mg/kg per d) beginning on postnatal day 7 was performed and compared with untreated BPK mice to determine whether long-term inhibition of 20-HETE formation would prolong the survival of the mice. Mice were monitored daily, and the date of mortality was noted. (A) Representative mouse kidney harvested at postnatal day 21 (left) from an untreated BPK mouse and postnatal day 46 (right) from a BPK mouse treated with HET-0016. (B) Graph demonstrating the survival of the untreated BPK mice and cystic BPK mice treated with HET-0016 (HET). The average days of survival are shown in the graph as means ± SEM; n = number of mice used in the experiment. **P < 0.0116 between the two groups using nonparametric Mann-Whitney U test.

Figure 3. Immunohistochemical analyses of cyst formation in the mouse kidney after long-term HET-0016 administration in vivo. (A and B) The localization of cyst development was determined in postnatal day 21 kidneys from BPK mice treated with vehicle (A) and long-term treatment with HET-0016 (10 mg/kg per d; B) beginning on postnatal day 7 and ending on day 20. As shown in A, large collecting tubule cysts, which were labeled with Dolichos biflorus agglutinin (DBA), a collecting duct lectin, were found in the BPK kidneys treated with vehicle. There was a dramatic reduction in the DBA-labeled collecting tubule cysts with a concomitant increase in the retention of Lotus tetragonolobus agglutinin (LTA) labeled proximal tubule (PT) cysts (brown), which is an indicator of early stages of cystic disease. Arrowheads point at noncystic PT, and thin arrows point at cystic PT.

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).

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 lentivector vectors expressed the EGFP transgene as determined by FACS analysis (Figure 6A), and no spu-

![Figure 4](https://www.jasn.org/jasn-web-data/basics-2008/fig4.jpg)

**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.

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 ± 32,171.0 (n = 4)</td>
<td>369,053 ± 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.

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 non-cystic 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 \((70\%)\) \((n = 2)\) after administration of HET-0016 at a dosage of 1 \(\mu\)M 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 (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).

**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 \(\mu\)M), 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 \(\mu\)M) relative to the vehicle-treated cells, indicating that an alternative eicosanoid may also play a
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 with 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.

Figure 7. Determination of the cell number in cystic BPK and noncystic Balb/c renal epithelial cells. (A) Cystic BPK and noncystic Balb/c renal epithelial cells were incubated in serum-free medium without γ-IFN at a temperature of 37°C (nongenomissive conditions) for a period of 6 d. At this point, the BPK (▲) and Balb/c (●) cells were plated into individual wells, and the cells were counted daily by hemocytometry over a 5-d period. HET-0016 (10 μM) was added to the medium on a daily basis for the 5-d period (n = 4 to 6 wells/time point). *P < 0.001 difference between BPK cells treated with VEH versus HET; †P < 0.001 difference between BPK (VEH) versus Balb/c treated with VEH or HET. (B) Noncystic Balb/c renal epithelial cells were serially transduced on a daily basis with lentiviral vectors expressing EGFP (○), Cyp4a10 (▲), and Cyp4a12 (□) under permissive conditions (in the presence of γ-IFN at 33°C). The cells were expanded and placed in nonpermissive conditions for a period of 6 d. At this point, the cells were plated into individual wells, and the cells were counted on a daily basis using a hemocytometer for a 5-d period (n = 4 to 6 wells/time point). HET-0016 (10 μM) was added to the medium with the cells transduced with the Cyp4a12-expressing lentiviral vector (●; n = 3 to 6 wells/time point). *P < 0.001 difference between CYP4A-transduced Balb/c compared with EGFP-transduced Balb/c cells.

Figure 8. Decreased cell-cycle progression in cystic BPK cells in the presence of 20-HETE synthesis inhibitors. Cystic BPK cells were incubated in serum-free medium without γ-IFN at a temperature of 37°C (nongenomissive conditions) for a period of 10 d and then incubated in the presence of vehicle (A) or two chemically dissimilar 20-HETE synthesis inhibitors, HET-0016 (10 and 20 μM; B and C) and DDMS (10 μM; D), for a period of 36 h. BrdU, which is a marker for DNA synthesis, was added at a concentration of 30 μM for 3 h before the end of the experiment. Cells were methanol-fixed, and cellular uptake of BrdU was identified by immunohistochemistry with biotinylated monoclonal anti-BrdU antibody. (D) Graphic representation of the data expressing the number of BrdU-labeled cells per 500 total cells counted. n = 2 to 4 samples/group. **P < 0.05 difference between HET-0016 versus vehicle-treated cystic BPK cells.
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 \(^{19-23,31}\); 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.\(^{31}\) 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 \(^{22-25}\); 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.\(^{26}\) 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,\(^{21}\) cirrhosis,\(^{27}\) and endothelial dysfunction.\(^{28}\)

Several reports have linked the 20-HETE–mediated release of AA and its subsequent proliferative response through an interaction with the EGF receptor using both normally quiescent \(^{12,13,29}\) and actively proliferating oncogenic cells.\(^{15,16}\) The interaction of the EGF receptor with 20-HETE may play an important role in PKD as a result of the well-established effect of EGF 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.\(^{12,19,23,24,30-32}\)

Recent work showed that 20-HETE has been found to promote angiogenesis \textit{in vivo} in a growth factor–dependent manner,\(^{33}\) and blockade of the vascular endothelial growth factor receptor abolished the 20-HETE mediated effects. Chen \textit{et al.}\(^{29}\) found that WIT003, a 20-HETE agonist, could induce mitogenesis in endothelial cells \textit{in vitro} and angiogenesis in the rat cornea \textit{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% \textit{in vivo}.\(^{29}\) Other studies using norepinephrine, AngII, and EGF have been shown to stimulate the synthesis and release of 20-HETE\(^{13,17}\) and promote increased thymidine incorporation in vascular smooth muscle cells\(^{14}\) and proximal tubular epithelial cells in the kidney\(^{15}\) through a Ras-dependent\(^{14,17}\) and -independent activation\(^{14}\) of the mitogen-activated protein kinase (MAPK) pathway.

More recent studies by Guo \textit{et al.}\(^{15,16}\) demonstrated an interaction between the EGF, 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 phospholipase A2 to release AA in vascular smooth muscle 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 proctoid 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 lenti-vascular 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. Mice were treated with vehicle or HET-0016 (10 mg/kg per d intraperitoneally) beginning on postnatal day 7 and killed on postnatal 21. 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 Immunobed 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 nephron 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 1× 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 TBS-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.20 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 polyurine tract sequence and the woodchuck postregulatory element as shown in Figure 5. For the cloning of the Cyp4a10-expressing transfer plasmid, pHR(+)cUb.GFP.R(-)W(+) was double digested with Kpnl and XbaI to remove the GFP cDNA and was replaced with the XbaI/Kpnl fragment containing the Cyp4a10 cDNA. The final construct was named pHR(+)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 pHR(+)cUb.Cyp4a12(short).R(−)W(+) plasmid to make the final construct, pHR(+)cUb.Cyp4a12.R(−)W(+).

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 dissimilatory inhibitors of cytochrome P450 4A (CYP4A) and 4F (CYP4F) inhibitors, specifically HET-0016 (10 and 20 μM) and DDMS (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 immunohistochemistry 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 RQ 1 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’-GACAGGACCTAGT-GCTGAGG-3’ and antisense 5’-CTCATAGAAATGTTCACA-3’; Cyp4a12 sense 5’-TGATGTCTTATGAAAGAATGGC-3’ and antisense 5’-CTGGAAGCAGCAGAAGGTG-3’ and Cyp4a14 sense 5’-CTCTAAGGTACTTGGATGTT-3’ and antisense 5’-ATACATAAAGGAGACTGTATA-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.

Lentiviral Vector Production

Modified lentiviral vectors were produced by transient triple-plasmid transfection of 293T cells as described previously.58-51 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 transfected 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.19,42 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.

Packaging and Envelope Pseudotype Plasmids

pCMVAβ8.74 is the packaging plasmid that provides the expression of the gag-pol, tat, and rev genes, and the viral accessory genes have been deleted or attenuated as described previously by Dull et al.46 pMD.G is the envelope plasmid and encodes the vesicular stomatitis virus G protein as described previously.47

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
assay, the isolated protein (500 µg) was incubated in 0.5 ml of 100 mM potassium phosphate buffer containing 40 µM cold AA and 2 mM NADPH. In some samples, the 20-HETE synthesis inhibitor HET-0016 was added at a concentration of 1 µM. The reaction was incubated at 37°C and equilibrated with 100% O2 for 30 min. The reaction was stopped by acidification to pH 3.5 with 1 M formic acid. The lipids were extracted in the presence of 20-HETE-d6 (2 ng) using 3:1 ethyl acetate:water and dried down under nitrogen. The samples were reconstituted in 1:1 methanol:water, and the production of 20-HETE was measured using a liquid chromatograph–triple quadrupole mass spectrometer (ABI 3000; Applied Biosystems, Foster City, CA) as described previously.53

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.

ACKNOWLEDGMENTS

This work was funded in part by a grant award from the Polycystic Kidney Disease (PKD) Foundation (F.P.), Advancing a Healthier Wisconsin award (E.D.A. and W.E.S.), National Institutes of Health HL 36279 (R.J.R.), and National Institutes of Health P50 DK057306 (E.D.A.).

We thank Averia Steinman and Katherine Friedrich for technical help in the sample isolation and 20-HETE production assay and Emma T. Schwasinger and Deborah L. Donahoe for technical help with the breeding and handling of the mice.

DISCLOSURES

None.

REFERENCES


