Hypoxia-inducible factor-1α (HIF-1α) and autophagy in polycystic kidney disease (PKD)

Franck Belibi, Iram Zafar, Kameswaran Ravichandran, Anamarija Bauer Segvic, Alkesh Jani, Danica Galesic Ljubanovic, and Charles L. Edelstein

1Division of Renal Diseases and Hypertension, University of Colorado at Denver and Health Sciences Center, Aurora, Colorado; and 2Medical School, Zagreb Dubrava University Hospital, Zagreb, Croatia

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Belibi F, Zafar I, Ravichandran K, Segvic AB, Jani A, Ljubanovic DG, Edelstein CL. Hypoxia-inducible factor-1α (HIF-1α) and autophagy in polycystic kidney disease (PKD). Am J Physiol Renal Physiol 300:F1235–F1243, 2011. First published January 26, 2011; doi:10.1152/ajprenal.00348.2010.—Cyst expansion in polycystic kidney disease (PKD) results in localized hypoxia in the kidney that may activate hypoxia-inducible factor-1α (HIF-1α). HIF-1α and autophagy, a form of programmed cell repair, are induced by hypoxia. The purposes were to determine HIF-1α expression and autophagy in rat and mouse models of PKD. HIF-1α was detected by immunoblotting. Eight-week-old male heterozygous (Cy+/+) and 4-wk-old cpk mice, and 112-day-old Pkd2WS25/− mice with a mutation in the Pkd2 gene were studied. HIF-1α was significantly increased in massive Cy/Cy and cpk kidneys and not smaller Cy/+ and Pkd2WS25/− kidneys. On EM, features of autophagy were seen in wild-type (+/+), Cy/+ and cpk kidneys: autophagosomes, mitophagy, and autolysosomes. Specifically, autophagosomes were found on EM in the tubular cells lining the cysts in cpk mice. The increase in LC3-II, a marker of autophagosome production and beclin, a regulator of autophagy, was greater in Cy/Cy and cpk kidneys, followed by a similar pattern of increase as HIF-1α. To determine the role of HIF-1α in cyst formation and/or growth, Cy/+ rats, Cy/Cy rats, and cpk mice were treated with the HIF-1α inhibitor 2-methoxyestradiol (2ME2). 2ME2 had a significant effect on kidney volume or cyst volume density. In summary, HIF-1α is highly expressed in the late stages of PKD and is associated with an increase in LC3-II and beclin-1. The first demonstration of autophagosomes in PKD kidneys is reported. Inhibition of HIF-1α did not have a therapeutic effect.

electron microscopy

AUTOSOMAL DOMINANT POLYCYSTIC kidney disease (ADPKD) is one of the most common hereditary diseases in the United States, with an estimated prevalence of 1 in 200–1,000 people (11). Over 50% of people with ADPKD develop chronic kidney disease that ultimately requires dialysis and renal transplantation (11). A better understanding of the pathophysiology of cyst formation may lead to the development of potential therapies to slow cyst formation or growth.

Cyst expansion in PKD kidneys results in localized areas of hypoxia. Evidence for the hypoxia in the kidney is the observation that PKD patients have significantly higher EPO levels than other end-stage renal disease (ESRD) patients (41). EPO gene expression is induced by hypoxia-inducible transcription factors (HIF) due to localized hypoxia in the cystic kidney. One study has demonstrated an increase in HIF-1α in humans and rat polycystic kidneys (4). Another study has shown increased expression of HIF-1α in polycystic livers (31). Hypoxic stimuli increase HIF-1α protein by inhibiting its degradation by the proteasome. Multiple studies in animal models of PKD have demonstrated activation of the mammalian target of rapamycin (mTOR) signaling pathway and the therapeutic effect of rapamycin in PKD (2, 14). Under both normoxic and hypoxic conditions, the phosphatidylinositol 3-kinase-mTOR pathway activates HIF-1α (9, 16, 22). The aim of the study was to determine HIF-1α expression in different models of PKD with varying severity of cyst formation.

HIF-1α is known to increase cell proliferation, angiogenesis, and apoptosis (9, 22). As proliferation of cyst lining epithelium and apoptosis play a causative role in cyst formation (2, 13), we also sought to determine the potential therapeutic effect of HIF-1α inhibition in PKD.

Autophagy has been described as a HIF-1α-dependent adaptive response (42). Autophagy is the process by which organelles and bits of cytoplasm are sequestered and subsequently delivered to lysosomes for hydrolytic digestion (23). Autophagy, also called “self-eating,” is a process of cell repair that usually accompanies apoptosis which is “self-killing” of the cell (19, 23). While apoptosis is a feature of most models of PKD including those for humans, rats, and mice (13), autophagy has not been described in PKD. In the current study, we sought to determine whether autophagy and its protein markers, LC3 and beclin-1, are upregulated in polycystic kidneys.

MATERIALS AND METHODS

Animal models. The study was conducted in homozygotes (Cy/Cy), heterozygous males (Cy/+), and normal littermate control male (+/+) Han:SPRD rats. Cy/Cy rats are diagnosed by palpable kidneys at 10–14 days of age. Cy/Cy rats develop massive polycystic kidneys with a 20-fold increase in two-kidney weight-to-total body weight ratio (2K/TBW) and die from renal failure at ~4 wk of age. Cy/+ rats develop a twofold increase in 2K/TBW and have mild renal failure at 8 wk of age. A colony of Han:SPRD rats was established in our animal care facility from a litter that was obtained from the Polycystic Kidney Program at the University of Kansas Medical Center.

cpk/+ mice in the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). The cpk mouse is a model of ARPKD. Thus heterozygous cpk mice (cpk+/+) do not have PKD, whereas homozygous cpk mice have massive polycystic kidneys with a 20-fold increase in 2K/TBW and usually die from renal failure at ~4 wk of age.

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wk of age. In this manuscript, the term “cpk” is used to refer to homozygous mice carrying two copies of the cpk gene (cystic cpk/cpk mice).

The study was also conducted in Pkd2WS25/− mice and normal littermate control (+/+ ) mice. The development of PKD and renal failure in Pkd2WS25/− mice has been described in detail (10, 38, 39). A colony of Pkd2WS25/− mice was established in our animal care facility from a litter that was obtained from Stefan Somlo at Yale University.

The study protocol was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee. Mice and rats had free access to tap water and standard mouse and rat chow.

Genotyping. The cpk gene encodes a hydrophilic, 145-amino acid protein termed cystin (15). In the cpk mouse, there is a tandem deletion of 12 and 19 bp in exon 1 of the cpk gene. cpk mutations are identified using a PCR primer set flanking the deletions. The following cpk exon I primer set amplified a 351-bp product from the wild-type cpk gene and a 320-bp product from a mutant cpk gene: 5′-CPK: 5′-TCC TCC CTC CTT ATC TCT CCA3′; 3′-CPK: 5′-ATC CAG CAG GCG TAG GGT CTC3′.

C57BL/6 Pkd2+/− and Pkd2WS25+/+ mice were used as breeding pairs to generate Pkd2WS25/− mice for the study. Mice were genotyped by Southern blotting (1, 39). Briefly, the genotype of ing pairs to generate Pkd2WS25/− mice is determined by hybridizing the PstI-digested tail DNA with a 32P-labeled probe (39). The size of the wild-type allele is 4.0 kb, the WS25 allele is 2.5 kb, and the null allele is 1.8 kb.

Cyst volume density. Hematoxylin-eosin-stained sections were used to determine the cyst volume density. This was performed by a reviewer blinded to the identity of the treatment modality, using point-counting stereology (8). At least 10 areas of the medulla at 90, 180, and 270° from the hilum of each section were selected to guard against field-selection variation.

**HIF-1α measurements.** HIF-1α was detected by electrochemiluminescence using an ultrasensitive singleplex kit from Meso Scale Discovery (MSD) as per the manufacturer’s instructions.

**Immunoblotting.** Immunoblot analysis was performed as we have previously described (33). The renal cortex was homogenized in lysis buffer (in mM: 5 Na₂HPO₄, 5 NaH₂PO₄, 150 NaCl, 1 EDTA, 0.1% Triton X-100, 50 NaF, and 0.2 Na₂VO₃, and 0.1% β-mercaptoethanol, pH 7.2) plus proteinase inhibitors 1 mM 4-(2-Morpholino)ethanesulfonic acid (MES), 10 mM pepstatin, 22 μM bestatin, 22 μM aprotinin, and 0.8 μM aprotinin. The homogenates were centrifuged (14,000 rpm at 4°C for 10 min) to remove unbroken cells and debris. Supernatants were mixed with sample buffer containing 50 mM Tris-base (pH 6.8), 0.5% glycerol, 0.01% bromophenol blue, and 0.75% SDS and heated at 95°C for 5 min. Equal amounts of protein (60 μg/lane) were fractionated by Tris-glycine-SDS–15% PAGE. The electrophoretically separated proteins were then transferred to a nitrocellulose membrane (Millipore, Bedford, MA) by wet electroblotting. The membranes were blocked with 5% nonfat dry milk in TBST [50 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween buffer, pH 7.5] overnight at 4°C. Immunoblot analyses were performed with the following antibodies: 1) an LC3 antibody (catalog no. 2775, Cell Signaling Technology, Beverly, MA) that detects endogenous levels of total LC3 protein; LC3 initially yields a 32-kDa protein; 2) a beclin-1 antibody (catalog no. NB500–249, Novus Biologicals, Littleton, CO) that detects levels of beclin-1 protein recognized as a 52-kDa protein; beclin-1 is an earlier marker that participates in the initiation and elongation processes of autophagosome formation; and 3) HIF-1α antibody (catalog no. NB100-134, Novus Biologicals).

**Immunofluorescence studies.** Kidney tissues were embedded in OCT, snap-frozen in liquid nitrogen, and stored at −80°C until sectioning. Five-micrometer cryostat sections were fixed in 70% acetone/30% methanol and prepared for immunofluorescence studies as described previously (26). The following primary antibodies were used: 1) LC3 (same antibody as described for immunoblot) and 2) HIF-1α antibody (catalog no. NB100-134, Novus Biologicals).

**Transmission electron microscopy.** For transmission electron microscopy (TEM) analysis, samples were fixed in McDowell fixative, washed in cacodylate buffer (pH 7.2), and postfixed in 2% osmium tetroxide in the same buffer. The samples were dehydrated in graded acetone series and embedded in agar epoxy resin. Semithin sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 400 transmission electron microscope at 100 kV.

**2-Methoxyestradiol and bafilomycin treatments.** Rats and mice were treated with the HIF-1α inhibitor 2-methoxyestradiol (2ME2; 2 mg·kg⁻¹·day⁻¹ i.p., product no. M6383, Sigma-Aldrich, St. Louis, MO) or vehicle (1% DMSO in PBS). The dose of 2ME2 is based on studies that demonstrate a protective effect of 2ME2 in rodent models of cerebral ischemia (8) and rheumatoid arthritis (27).

Mice were treated with bafilomycin A1 (2 mg·kg⁻¹·day⁻¹ i.p., Wako Chemicals, Richmond, VA) or vehicle (10% ethanol in normal saline) for 4 days and then killed for determination of LC3-II.

**Statistical analysis.** Nonnormally distributed data were analyzed by a nonparametric unpaired Mann-Whitney test. Multiple group comparisons were performed using ANOVA with a Newman-Keuls posttest. A P value <0.05 is considered statistically significant. Values are expressed as means ± SE.

**RESULTS**

Upregulation of HIF-1α in Cy/Cy and cpk kidneys. We determined whether HIF-1α is increased in whole kidneys of rats and mice with PKD using an ultrasensitive singleplex kit from MSD. The HIF-1α contents of different rodent species were compared (Table 1). Despite the fact that the 2K/TBW increase was found in Cy/+ rats and Pkd2WS25/− mice, levels of HIF-1α in Cy/+ and Pkd2WS25/−/− were not statistically different from the levels in +/+ rats. Interestingly, we found large increases in HIF-1α in massive kidneys from Cy/Cy rats and cpk mice compared with their respective +/+ rats and mice (Table 1). These results in three different models of PKD establish that HIF-1α is increased in late stages of PKD when the kidneys are massive.

Localization of HIF-1α in cyst-lining epithelial cells. To determine the localization of HIF-1α in PKD kidneys, immunofluorescence was performed. Cystic kidneys, from +/+ , Cy/+ , and Cy/Cy rats were stained and analyzed by confocal microscopy. Cells lining the cysts in Cy/+ and Cy/Cy kidneys showed intense HIF-1α staining. A representative picture of HIF-1α staining from at least three separate experiments is shown in Fig. 1.

**Autophagy.** To visualize autophagy in PKD kidneys, electron microscopy was performed. Electron micrographs of autophagosomes, mitochondria in a autophagosome (mitophagy), autophagosomes fusing with lysosomes, and autophagosomes in the epithelial cells lining cysts in +/+ , Cy/+ , and Cy/Cy rats and cpk mice are demonstrated in Fig. 2, A–N.
Table 1. 2K/TBW ratio and HIF-1α in kidneys of Cy/Cy rats and cpk mice

<table>
<thead>
<tr>
<th>Han:SPRD rats (n = 6/group)</th>
<th>8-Wk-old +/+</th>
<th>8-Wk-old Cy/+</th>
<th>4-Wk-old Cy/Cy</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>2K/TBW, %</td>
<td>0.9 ± 0.1</td>
<td>1.8 ± 0.1*</td>
<td>17.8 ± 2.2*</td>
<td>*P &lt; 0.001 vs. +/+</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>54.8 ± 4.2</td>
<td>52.3 ± 3.3</td>
<td>88.4 ± 3*</td>
<td>*P &lt; 0.001 vs. +/+</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Cpk mice (n = 7/group)</th>
<th>4-Wk-old +/+</th>
<th>4-Wk-old cpk</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2K/TBW, %</td>
<td>1.3 ± 0.1</td>
<td>28.7 ± 1.9*</td>
<td>*P &lt; 0.001 vs. +/+</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>62.5 ± 4.5</td>
<td>133 ± 11*</td>
<td>*P &lt; 0.001 vs. +/+</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Pkd2WS25/− mice (n = 4/group)</th>
<th>112-Day-old +/+</th>
<th>112-Day-old Pkd2WS25/−</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2K/TBW, %</td>
<td>1.5 ± 0.1</td>
<td>2.0 ± 0.2*</td>
<td>*P &lt; 0.01 vs. +/+</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>59.7 ± 3.0</td>
<td>62.7 ± 5.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. 2K/TBW, 2-kidney weight-to-total body weight ratio; HIF-1α, hypoxia-inducible factor-1α; NS, not significant. Kidney size, as indicated by 2K/TBW ratio, was massively increased in Cy/Cy rats and cpk mice compared with normal controls (+/+). HIF-1α was increased in Cy/Cy rat and cpk mouse kidneys.

cytosolic form LC3-I, which is converted to LC3-II during the formation of autophagosomes (23). LC3-II is the only known protein that is recruited to both inner and external surfaces of the expanding autophagosomal membranes and specifically associates with autophagosomes and not with any other vesicular structures.

Rats and mice were treated with bafilomycin A1 (2 mg·kg\(^{-1}\)·day\(^{-1}\) ip) or vehicle for 4 days. Bafilomycin at a dose of 2 or 1 mg·kg\(^{-1}\)·day\(^{-1}\) for 4 days resulted in the death of +/+ and Cy/Cy rats, and +/+ and cpk mice treated bafilomycin A1 (2 mg·kg\(^{-1}\)·day\(^{-1}\) for 4 days) well. The intensity of the LC3-II bands was increased in cpk mice compared with +/+ mice (Fig. 3A). In +/+ mice treated with bafilomycin A1, there was an increase in LC3-II compared with vehicle-treated +/+ mice (Fig. 3A). In cpk mice treated with bafilomycin A1, LC3-II was not increased compared with vehicle-treated cpk mice (Fig. 3A). LC3-II was increased in Cy/Cy rats compared with +/+ and Cy/+ rats (Fig. 3B). β-Actin used as a loading control was not different between the groups (*P < 0.05 vs. controls; n = 4/group).

While increased LC3-II was seen on immunoblots in Cy/Cy, autophagosomes were not visualized on electron microscopy (Fig. 2, H and I) in Cy/Cy. The reason for the discrepancy between LC-3 on immunoblots and autophagosomes on electron microscopy is not clear. However, it is known that electron microscopy is not a good technique for quantifying autophagosomes (23).

Beclin-1 is the mammalian ortholog of the yeast Apg6/Vps30 gene that participates in the initiation and elongation processes of autophagosome and has a key role in autophagy (23) (19) (37). In agreement with the LC3-II findings, increased beclin-1 expression was observed in cpk mice.
and Cy/Cy rats (Fig. 4B) compared with respective normal littermate controls (+/+). These results demonstrate that beclin-1 is upregulated in PKD and may be a critical regulator of autophagy in PKD.

**Localization of LC3 in cyst-lining epithelial cells.** To characterize the distribution of endogenous LC3 in cystic kidneys, we performed immunofluorescent analysis. In agreement with the HIF-1α findings, cells lining the cysts showed intense staining in Cy/+ and Cy/Cy (Fig. 5). Staining was mainly in the cytoplasm. A representative picture of LC3 staining from at least three separate experiments is shown in Fig. 5.

**HIF-1α inhibition.** HIF-1α is significantly increased in kidneys from Cy/Cy rats and cpk mice (Table 1) and in the cells lining the cysts in Cy/Cy rats (Fig. 1). To determine the potential functional role of HIF-1α, inhibition studies were performed.

Cy/Cy rats were identified by the palpation of polycystic kidneys at day 14 of age. Rats were treated with the 2ME2 (2 mg·kg⁻¹·day⁻¹ ip) or vehicle from 14 to 28 days of age. Cy/Cy rats usually die from renal failure due to massively enlarged kidneys at ~day 28 of age. Both vehicle- and 2ME2-treated Cy/Cy rats became very sick at days 27–28 of age, and the kidneys were removed just before death. Immunoblotting of Cy/Cy kidneys demonstrated a decrease in HIF-1α protein in 2ME2-treated rat kidneys compared with vehicle-treated rats (Fig. 6). However, the 2K/TBW ratio and cyst volume density

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**Fig. 2.** Electron microscopy of autophagy. A–C: +/+ rats. Magnification ×2,500 (A), ×12,000 (B), and ×20,000 (C). D–G: Cy/+ rats. Magnification ×2,500 (D), ×40,000 (E), ×40,000 (F), and ×60,000 (G). H and I: Cy/Cy rats. Magnification ×2,500 (H) and ×8,000 (I). J and K: +/+ mice. Magnification ×2,500 (J) and ×6,000 (K). L–N: cpk mice. Magnification ×2,500 (L), ×12,000 (M), and ×12,000 (N). Autophagosomes (arrows) are demonstrated in kidneys of +/+ rats (A–C), Cy/+ rats (D–G), +/+ mice (J and K), and cpk mice (L–N). Lysosomes (L) fusing to autophagosomes are shown in F and G. Mitochondria (M) within a autophagosome (mitophagy) are shown in C and F. Autophagosomes were not seen in epithelial cells lining cysts in Cy/Cy rats (arrowheads, H and I). Autophagosomes in epithelial cells lining cysts are shown in J–L.
were not different in 2ME2- vs. vehicle-treated Cy/Cy rats (Fig. 6).

+/+ and Cy/+ rats were also treated with the HIF-1α inhibitor 2ME2. Cy/Cy rats were identified by the palpation of polycystic kidneys at day 14 of age and removed from the litters. The rest of the litter consisting of +/+ and Cy/+ rats were treated with 2ME2 (2 mg·kg$^{-1}·$day$^{-1}$ ip) or vehicle for 5 wk from 4 to 8 wk of age. The blood urea nitrogen, 2K/TBW ratio, and cyst volume density were not different in 2ME2- vs. vehicle-treated Cy/+ rats (Table 2).

Cpk mice were identified by the palpation of polycystic kidneys at day 14 of age. Mice were treated the HIF-1α inhibitor 2ME2 (2 mg·kg$^{-1}·$day$^{-1}$ ip) or vehicle from 14 to 21 days of age. Cpk mice usually die from renal failure due to massively enlarged kidneys at days 21–28 of age. Both vehicle- and 2ME2-treated cpk mice became very sick at day 21 of age, and the kidneys were removed just before death. Immunoblotting of cpk kidneys demonstrated a decrease in HIF-1α protein in 2ME2-treated mouse kidneys compared with vehicle-treated mice (Fig. 7). However, the 2K/TBW ratio and cyst volume density were not different in 2ME2- vs. vehicle-treated cpk mice (Fig. 7).

**DISCUSSION**

The first aim of the study was to examine HIF-1α expression in kidneys with varying degrees of cyst formation. In kidneys from Cy/+ rats and PkdWS25/− mice that have a small but significant increase in kidney volume-to-body weight ratio, there was no increase in HIF-1α protein expression in whole kidney homogenates. However, on immunofluorescence, the cyst epithelial cells in Cy/+ rat kidneys did show HIF-1α staining. In kidneys from Cy/Cy rats and cpk mice that have a massive ~20-fold increase in kidney volume-to-body weight ratio, there was a large increase in HIF-1α protein. In addition, HIF-1α staining on immunofluorescence was present in epithelial cells lining large cysts in Cy/Cy kidneys. On the one hand, the presence of a large increase in HIF-1α in very large cystic kidneys suggests that the increase in HIF-1α is a result of more localized hypoxia with larger cysts rather than the cause of the cyst expansion. The presence of regional areas of hypoxia around large cysts has been demonstrated (4). On the other hand, HIF-1α expression was seen in epithelial lining cells of smaller cysts in Cy/+ animals, suggesting that HIF-1α could be the cause of the cyst expansion and may increase pericystic angiogenesis.

To determine whether HIF-1α may be playing a causative role in cyst expansion, inhibition studies were undertaken. HIF-1α induces genes that play a role in many cellular functions including cell proliferation, angiogenesis, and apoptosis (9, 22). Increased cell proliferation of cyst-lining epithelium plays a causative role in cyst growth (2). Increased angiogenesis is a feature of polycystic kidneys (35). The role of apoptosis in PKD is controversial (13). It has been shown that caspase and apoptosis inhibition is protective in PKD (34). However, it has also been shown that the protective effect of rapamycin in PKD is associated with increased apoptosis in cysts (29, 30). Thus HIF-1α inhibition may be protective by inhibitory effects on cell proliferation, angiogenesis and apoptosis. 2ME2 is an endogenous metabolite of estrogen and has antiproliferative, antiangiogenic, and antitumor effects (20, 24). 2ME2 has disease-modifying effects, attributable to the inhibition of neovas-
cular events, in a mouse model of rheumatoid arthritis (27). 2ME2 also has antiarthritis properties by inhibiting inflammation independently of its antiangiogenic properties (17). 2ME2 attenuates the hemorrhagic conversion of cerebral infarcts in rats (8). 2ME2 or its analogs inhibit tumor growth in mice (20, 25, 28). 2ME2 has been well tolerated by patients in phase I and II clinical studies (24). In the pilot study, Cy/Cy rats and cpk mice that have a significant

![Immunoblotting for LC3-II](image)

Fig. 3. Immunoblotting for LC3-II. LC3-II specifically associates with autophagosomes and not with any other vesicular structures. The intensity of the LC3-II bands was increased in cpk mice compared with wild-type (+/+) mice (A). In +/+ mice treated with bafilomycin A1, there was an increase in LC3-II (A). In cpk mice treated with bafilomycin A1, LC3-II was not increased (A). LC3-II was increased in Cy/Cy rats compared with +/+ and Cy/+ rats (B) compared with respective normal littermate controls. β-Actin, used as a loading control, was not different among the groups. *P < 0.05 vs. controls; n = 4/group.

![Immunoblotting for beclin-1](image)

Fig. 4. Immunoblotting for beclin-1. Beclin-1 participates in the initiation and elongation processes of autophagosome and has a key role in autophagy. The intensity of the beclin-1 bands was increased in cpk mice (A) and Cy/Cy rats (B) compared with respective normal littermate controls. β-Actin, used as a loading control, was not different between the groups. *P < 0.05 vs. controls; n = 4/group.
increase in HIF-1α in their polycystic kidneys were treated with 2ME2. Inhibition of HIF-1α in the polycystic kidney by the 2 mg·kg⁻¹·day⁻¹ dose of 2ME2 was demonstrated. However, 2ME2 did not have a significant effect on kidney volume or cyst volume in Cy/Cy rats and cpk mice. It should be noted that treatment was started at a late stage of the disease when cyst formation was advanced and that the pilot studies were undertaken in rats and mice with severe disease. Thus a 5-wk treatment study in Cy/+ rats with milder PKD was performed. 2ME2 had no effect on blood urea nitrogen, 2K/TBW ratio, or cyst volume in Cy/+ rats compared with +/+ rats. The rats and mice tolerated 2ME2 without visible side effects.

Autophagy describes the process by which cytoplasmic materials including organelles reach the lysosomes for degradation (23). Thus autophagy exerts cytoprotective effects. As hypoxia, apoptosis, and mTOR signaling are modulators of autophagy (13) (18) and are also features of PKD kidneys, we determined the effect of PKD on autophagy. Autophagy occurs constitutively at low levels and plays an essential role under physiological conditions, e.g., maintenance of the amino acid pool during starvation, antiaging, regulation of innate and adaptive immunity (23). This likely explains the presence of autophagy seen on EM in normal rat and mouse kidneys (Fig. 2, A–C, J, and K). Although autophagosomes cannot be quantified by TEM, this technique provides an insight into the extent of ongoing autophagy in cells (23). As quantitation of autophagy on EM is unreliable (23), we measured LC3-II. LC3-II is the cleaved isoform of LC3 that localizes to autophagosomes and phagolysosomes during autophagy (37). LC3-II was increased in massive Cy/Cy and cpk kidneys compared with littermate controls. The amount of LC3-II at a specific time may represent either increased autophagy or suppression of downstream steps, e.g., autophagosome-lysosome fusion. “Autophagic flux” is a term used to describe the dynamic process of autophagosome synthesis, delivery of autophagosomes to the lysosome, and degradation of autophagosomes in the lysosome (23). To measure autophagic flux, lysosomal in-

**Fig. 5.** Immunofluorescence for LC3. To demonstrate that LC3 is present in the cells lining the cysts, immunofluorescence was performed. There is LC3 staining (red) in cells lining the cysts in Cy/+ and Cy/Cy rats. Nuclei are represented by blue (DAPI) staining. LC3 staining is cytosolic.

**Fig. 6.** 2-Methoxyestradiol (2ME2) treatment in Cy/Cy rats. Cy/Cy rats were treated with the HIF-1α inhibitor 2ME2 or vehicle from 14 to 28 days of age. Kidneys were removed at death for determination of HIF-1α protein, kidney size as determined by 2K/TBW ratio, and cyst volume density (CVD). 2ME2 treatment (2 mg·kg⁻¹·day⁻¹ ip) resulted in inhibition of HIF-1α as determined by immunoblotting. There was no significant difference in 2-kidney-to-total body weight (2K/TBW) ratio and CVD between vehicle- and 2ME2-treated Cy/Cy rats. NS, not significant; n = 5/group.
HIF-1α AND AUTOPHAGY IN PKD

Table 2. Effect of 2ME2 treatment on 2K/TBW, cyst volume density, and BUN in Cy/+ rats

<table>
<thead>
<tr>
<th></th>
<th>+/+ Vehicle</th>
<th>+/+ 2ME2</th>
<th>Cy/+ Vehicle</th>
<th>Cy/+ 2ME2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2K/TBW, %</td>
<td>0.8</td>
<td>0.8</td>
<td>1.8*</td>
<td>2*</td>
</tr>
<tr>
<td>CVD</td>
<td>1</td>
<td>2</td>
<td>30.3*</td>
<td>27.4*</td>
</tr>
<tr>
<td>BUN</td>
<td>21</td>
<td>18</td>
<td>30*</td>
<td>28*</td>
</tr>
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2ME2, 2-methoxyestradiol; CVD, cyst volume density; BUN, blood urea nitrogen. 2ME2 treatment had no effect on 2K/TBW, CVD, and BUN in Cy/+ rats (n = 4-5/group). *P < 0.05 vs. +/- vehicle.

Fig. 7. 2ME2 treatment in cpk mice. Cpk mice were treated with the HIF-1α inhibitor 2ME2 or vehicle from 14 to 21 days of age. Kidneys were removed at death for determination of HIF-1α protein, kidney size as determined by 2K/TBW ratio, and CVD. 2ME2 treatment (2 mg·kg⁻¹·day⁻¹ ip) resulted in inhibition of HIF-1α as determined by immunoblotting. There was no significant difference in 2K/TBW ratio and CVD between vehicle- and 2ME2-treated cpk mice; n = 3/group.

Autophagy is a cellular process that allows cells to degrade damaged proteins, organelles, and cellular debris. In PKD, autophagy plays a crucial role in disease progression. Increased autophagy has been observed in PKD kidneys, suggesting that it may play a causative role in the disease. One key regulator of autophagy is the protein HIF-1α, which is a transcription factor that is activated in response to hypoxia or other stress conditions. HIF-1α stimulates the expression of genes involved in various cellular functions, including autophagy.

In wild-type mouse kidneys, LC3-II was increased in the presence of bafilomycin A1, suggesting increased autophagic flux. LC3-II was increased in cpk compared with wild-type kidneys. The increase in LC3-II in PKD vs. wild-type kidneys suggests either increased autophagosome synthesis in PKD or decreased degradation in the lysosome in PKD. To investigate whether there was increased autophagosome synthesis or decreased degradation in the lysosome, LC3-II was increased in the cpk kidneys. The lack of effect of bafilomycin A1 on LC3-II in cpk kidneys suggests a defect in autophagy in PKD resulting from a block of autophagosome-lysosome fusion and degradation.

What is the significance of autophagy in PKD? A major function of autophagy is to keep cells alive under “stressful” conditions. Increased apoptotic cell death in the tubular cells lining the cyst plays a role in disease progression in PKD (13). Autophagy in the tubular epithelial cells lining the cysts may be a response to the cell death in these cells. Alternatively, autophagy suppression is associated with certain diseases, e.g., cancer, and is a feature of aging. Bafilomycin resulted in an increase in LC3-II in wild-type but not in PKD kidneys. Thus autophagy suppression may play a role in disease progression in PKD. Further study of autophagy in PKD is merited especially in view of the fact that apoptosis plays an important role in disease progression in PKD and that there is important cross talk between apoptosis and autophagy (6, 21). Also, mTOR activation inhibits autophagy and mTOR activation is a feature of PKD (32, 40).

Beclin-1 (also known as atg6/Beclin 1) interacts with various anti-apoptotic proteins, and has recently been found to be a beclin-2-homology-3-only protein. Beclin-1 regulates both formation and maturation of autophagosomes (23). Overexpression of beclin-1 in human MCF7 breast carcinoma cells promotes autophagy and inhibits in vitro tumorigenesis in nude mice (7). Heterozygous beclin-1 +/- mice have reduced autophagy levels and increased incidence of spontaneous tumors (7). These studies clearly demonstrate a causative role for beclin-1 in autophagy. Beclin-1 was increased in Cy/Cy and cpk kidneys, suggesting that it may play a causative role in the autophagy in PKD.

Next, we considered the connection between HIF-1α and autophagy. Both HIF-1α and LC3-II were increased in the whole kidney homogenates and in the cells lining the cyst in the same Cy/Cy and cpk kidneys. HIF-1α has been shown to upregulate both apoptosis and autophagy (3, 5). In this regard, we have demonstrated increased apoptosis in both Cy/Cy kidneys (12, 33) and cpk kidneys (36). Silencing of HIF-1α in chondrocytes results in decreased beclin-1 and decreased autophagy, suggesting that increased HIF-1α can cause autophagy (3, 5). Thus it is possible that the increased HIF-1α in the present study may play a causative role in the autophagy seen in PKD.

In summary, we report the first demonstration of autophagosomes in PKD kidneys. HIF-1α is highly expressed in the late stages of PKD in cpk mice and Cy/Cy rats. The increase in HIF-1α is associated with an increase in LC3-II and beclin-1. HIF-1α inhibition with 2ME2 did not significantly reduce kidney size in Cy/+ rats, Cy/Cy rats, and cpk mice.
REFERENCES


