Caspase-3 Gene Deletion Prolongs Survival in Polycystic Kidney Disease

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ABSTRACT
Pan-caspase inhibition reduces tubular apoptosis and proliferation and slows progression of disease in a rat model of polycystic kidney disease (PKD). It is unknown, however, which specific caspases are involved in PKD progression. Because caspase-3 is a major mediator of apoptosis, its role in autosomal recessive PKD was determined. Mice with caspase-3 gene deletion were crossed with mice harboring the congenital polycystic kidney (cpk) mutation to generate double-mutant mice. cpk;casp3/H11001/H11002 mice lived nearly 4 times longer than littermate control cpk mice (mean survival of 117 d versus 32 d, \( P < 0.01 \)), and cpk;casp3/H11001/H11002 mice lived slightly longer than controls (mean survival of 56 d). In addition, the kidney weight, relative to body weight, was significantly lower in the cpk;casp3/H11002 mice than in the cpk and cpk;casp3/H11001 mice. Despite deletion of caspase-3, however, apoptosis occurred and cysts formed; therefore, the alternative pathways of apoptosis in cystic kidneys were investigated. Caspase-7 was up-regulated and the anti-apoptotic protein Bcl-2 was down-regulated in cpk, cpk;casp3/H11001, and cpk;casp3/H11002 mice compared with wild-type controls. In summary, homozygous deletion of caspase-3 markedly prolongs survival of cpk mice, but a caspase-7-mediated pathway may compensate for the deficiency of functional caspase-3. These findings suggest that pan-caspase inhibition may have a greater therapeutic effect than selective caspase inhibition in PKD.


Inherited polycystic kidney disease (PKD) is one of the leading causes of end-stage kidney disease requiring dialysis and kidney transplantation in children and adults.\(^1\) Inherited PKD includes both autosomal dominant and autosomal recessive forms. Autosomal dominant polycystic kidney disease (ADPKD) results from mutations in one of two genes, PKD1 or PKD2. The prevalence of ADPKD varies between 1 in 400 and 1 in 1000, thus making it one of the most common hereditary diseases in the United States. ADPKD progresses to end-stage renal disease (ESRD) over a period of decades in 50% to 75% of affected people. Autosomal recessive polycystic kidney disease (ARPKD) results from a mutation in a single gene, PKHD1. ARPKD is less common, affecting about 1 in 20,000 live births and results in ESRD in childhood.\(^2\)

The congenital polycystic kidney (cpk) mouse is the most extensively characterized mouse model of PKD.\(^3\) The inheritance, cyst localization in the kidney, and severity of kidney disease in the cpk mouse resembles ARPKD. Cys1, the cpk gene, encodes a cilia-associated protein called cystin that is disrupted in the cpk mouse.\(^4\) Increased apoptosis in polycystic kidneys has been described in cpk mice\(^1–8\) as well as in human and other rat and mouse models of PKD.\(^9\) In support of a deleterious effect of apoptosis in PKD, we have recently demonstrated that pan-caspase inhibition reduces tubular apoptosis and proliferation and slows disease...
progression in the Han:SPRD rat model of PKD. The effect of caspase or apoptosis inhibition in other rat and mouse models of PKD is not known. Caspase-3 is the major mediator of apoptosis (i.e., the so-called “executioner” caspase) and caspase-1 is a pro-inflammatory caspase. However, the effect of inhibition of a specific caspase on PKD is not known. We tested the hypothesis that specific inhibition of caspase-3 would prolong life and reduce cyst formation in PKD.

In the present study, we developed cpk mice that were either heterozygous or homozygous for caspase-3 deletion to determine the effect of specific caspase-3 deletion on the development of PKD.

RESULTS

Genotyping of the cpk and caspase-3 alleles

Genomic DNA samples were prepared from the tails of tested mice. The genotyping of the cpk and caspase-3 allele is demonstrated in Figure 1.

![Figure 1](image)

**Figure 1.** Genotyping of the cpk and caspase-3 allele. The normal and mutated cpk and caspase-3 alleles were amplified by PCR using primers described in “Methods.” For the caspase-3 PCR, the 320 bp band corresponds to normal allele and the 300 bp band corresponds to mutated allele. For the cpk PCR, the 351 bp band corresponds to normal allele and the 320 bp band corresponds to mutated allele.

Caspase-3 protein expression in the kidney

There was an increase in caspase-3 protein expression in cpk mice compared with normal control +/+ mice (Figure 2). Caspase-3 expression was decreased in cpk;casp3−/− mouse kidneys. The cpk;casp3+/− mice that had the longest survival (105 d and 113 d) had less expression of caspase-3 than the cpk;casp3−/− mice that had the shortest survival (29 d and 17 d). Caspase-3 expression was absent in cpk;casp3−/− mouse kidneys.

Effect of caspase-3 gene deletion on the development of PKD

Representative kidney sections, stained with hematoxylin and eosin, at the same magnification are demonstrated in Figure 3A. The two-kidney/total body weight ratio (2K/TBW %) was significantly decreased in cpk;casp3−/− mice compared with cpk. Mean 2K/TBW (%) was 25 ± 5 in cpk and 13 ± 3 in cpk;casp3−/− (P < 0.05 versus cpk). Figure 3B shows the individual data points for 2K/TBW. The cyst volume density (CVD) was the same when comparing 32-d-old cpk mice and 117-d-old cpk;casp3−/− mice. Mean CVD (%) was 0.7 ± 0.1 in +/+; 71 ± 4 in cpk (P < 0.001 versus +/+), and 65 ± 3 in cpk;casp3−/− (P < 0.001 versus +/+). Figure 3C shows the individual data points for CVD. The serum creatinine was the same when comparing 32-d-old cpk mice and 117-d-old cpk;casp3−/− mice. Serum creatinine (mg/dl) was 0.3 ± 0.01 in +/+; 1.3 ± 0.1 in cpk (P < 0.001 versus +/+), and 1.35 ± 0.05 in cpk;casp3−/− (P < 0.001 versus +/+). Figure 3D shows the individual data points for serum creatinine. Homozygous caspase-3 gene deletion markedly prolonged survival. Mean survival (days) was 32 ± 6 in cpk mice and 117 ± 17 in cpk;casp3−/− mice (P < 0.01 versus cpk). Figure 3E shows the individual data points for survival.

The survival data in the cpk;casp3−/− mice was variable. This variability may be related to the degree of caspase-3 expression (Figure 2). In the cpk;casp3+/−, the mean 2K/TBW (%) was 27 ± 1 and the 2K/TBW (%) for each animal studied was 31, 29, 26, 26, and 23. In the cpk;casp3+/−, the mean CVD (%) was 68 ± 3 and the CVD (%) for each animal studied was 72, 71, 69, 65, and 63. Blood for serum creatinine measurement was not available in cpk;casp3−/− mice. In the cpk;casp3+/−, the mean survival (days) was 56 ± 21 in cpk;casp3+/− and the survival (days) for each animal studied was 113, 105, 29, 17, and 17.

Kidney palpation was performed on all mice at 30 d of age. The two cpk;casp3+/− mice that survived 113 and 105 d and the cpk;casp3−/− mice had smaller kidneys on palpation than cpk mice at 30 d of age.

Apoptosis in PKD kidneys

The number of apoptotic (terminal deoxynucleotidyltransferase (TdT) mediated dUTP nick-end labeling (TUNEL)-positive) cells per high power field (HPF) in tubular cells and interstitium was not significantly different in cpk;casp3+/− and cpk;casp3−/− mice compared with cpk mice (Figure 4A). A rep-
A representative picture of the TUNEL-positive cells in the kidney is demonstrated in Figures 4B, C, and D for cpk, cpk;casp3/H11001/H11002, and cpk;casp3/H11002/H11002, respectively.

Apoptotic pathways in casp3−/− mice
Caspase-3 is the major mediator of apoptosis. Because there was apoptosis and PKD in the absence of caspase-3, alternative pathways of apoptosis were investigated. Caspase-7, like caspase-3, is an “executioner” caspase. There was an increase in caspase-7 protein expression in the kidney in cpk, cpk;casp3/H11001/H11002, and cpk;casp3/H11002/H11002 mice compared with +/+ mice (Figure 5A). Anti-apoptotic Bcl-2 protein expression was decreased in cpk, cpk;casp3/H11001/H11002, and cpk;casp3/H11002/H11002 mice compared with +/+ mice (Figure 5B). Pro-apoptotic Bax protein expression was unchanged in these mice (Figure 5C).

DISCUSSION
ADPKD is one of the most common life-threatening hereditary disorders. ADPKD is more common than sickle cell disease, cystic fibrosis, muscular dystrophy, hemophilia, Down syndrome, and Huntington disease combined. Considerable basic and clinical research is underway to find a specific treatment for PKD. There is increased proliferation of cystic tubular epithelial cells in human PKD and in animal models of PKD. Therapeutic agents that reduce cyst epithelial cell proliferation have shown promise in reducing cyst formation in animal models of PKD. These agents include lovastatin, EGF receptor tyrosine kinase inhibitors, rapamycin, c-myc antisense, and most recently the cyclin-dependent kinase inhibitor roscovitine. The vasopressin-2 receptor antag-
In the present study, we investigated the effect of specific ischemia because of decreased apoptotic cell loss in ischemic PKD. The specific caspase inhibitor IDN-8050 reduces tubular apoptosis and proliferation was decreased in cpk/casp3−/− mice compared with +/+ mice. Anti-apoptotic Bcl-2 protein (26 kDa) expression was decreased in cpk, cpk/casp3−/−, and cpk/casp3−/− mice compared with +/+. Pro-apoptotic Bax protein (23 kDa) expression was unchanged. Representative immunoblots of at least 3 separate experiments.

We have previously demonstrated that the pan-caspase inhibitor IDN-8050 reduces tubular apoptosis and proliferation and slows disease progression in the Han:SPRD rat model of PKD. IDN-8050 is a pan-caspase inhibitor that inhibits both pro-apoptotic caspases, such as caspase-3, -7, -8, and -9 as well as proinflammatory caspases, such as caspase-1 (formerly known as ICE (IL-1 converting enzyme)). The specific caspase that mediates apoptosis in PKD is not known. There are multiple rat and mouse models of both ADPKD and ARPKD. Besides, in the Han:SPRD rat model of ADPKD, the effect of caspase and apoptosis inhibition in other models of PKD is not known. Demonstration of an effect of caspase inhibition in more than one model of PKD may highlight the potential therapeutic benefit of either pan-caspase or specific caspase inhibition in human PKD.

Caspsase-3−/− mice bred on a C57BL/6 background survive until adulthood and are protected against in vivo cerebral ischemia because of decreased apoptotic cell loss in ischemic brain. In the present study, we investigated the effect of specific caspase-3 inhibition in PKD by crossing the caspase-3−/− mouse with the cpk/+ mouse to develop double-knockout mice. In the present study, the cpk mouse died from renal failure and PKD at a mean age of 32 d. Complete deficiency of caspase-3 remarkably prolonged the life of the cpk mouse. In one other study using the microtubule specific agent Taxol has the survival of cpk mice been markedly prolonged.

In a recent study in cpk mice, administration of the cdk inhibitor roscovitine from 7 to 21 d of age resulted in approximately a 25% reduction in kidney size, cyst volume, and renal function at 21 d of age. In this study, survival was not determined.

We have previously described the activation of caspase-3 and dysregulation of the balance between pro- and anti-apoptotic Bcl-2 family proteins in the Han:SPRD rat model of PKD. In the present study, at the time of demise, the double-knockout mice eventually had as much apoptosis in the polycystic kidneys as cpk mice, but these cpk/casp3−/− mice died after a markedly prolonged life span. Compensatory activation of caspase-7 has been reported when caspase-3 is deficient or absent. Thus, we investigated alternative pathways of apoptosis (Figure 6). Caspases-3 and -7 have some similar but also distinct roles in apoptosis. Caspase-7 is thought to mediate DNA fragmentation and the morphologic changes of apoptosis. Caspase-7 may be more important in the loss of cellular viability either alone or in combination with caspase-3. We demonstrate that, in the absence of caspase-3, the levels of caspase-7 protein in the kidney in caspase-3-deficient mice are as high as in the cpk mice. The activation of caspase-3 is known to be controlled by the balance between pro- and anti-apoptotic Bcl-2 family proteins via cytochrome c release from mitochondria, which results in activation of caspase-9. The effect of the balance between pro- and anti-apoptotic Bcl-2 family proteins on caspase-7 is less well described. We studied the anti-apoptotic protein Bax. Bcl-2 protein expression was decreased in both cpk mice and caspase-3-deficient cpk mice compared with normal control mice. The pro-apoptotic protein Bax remained unchanged. These findings suggest that the partial or complete absence of caspase-3 in polycystic kid-

![Figure 5](image-url). Immunoblots of caspase-7, Bcl-2, and Bax proteins. Full-length caspase-7 protein expression (35 kDa) in the kidney was increased in cpk, cpk/casp3−/−, and cpk/casp3−/− mice compared with +/+ mice. Anti-apoptotic Bcl-2 protein (26 kDa) expression was decreased in cpk, cpk/casp3−/−, and cpk/casp3−/− mice compared with +/+ mice. Pro-apoptotic Bax protein (23 kDa) expression was unchanged. Representative immunoblots of at least 3 separate experiments.

![Figure 6](image-url). Pathways of caspase-mediated apoptosis. In the “mitochondrial” or “intrinsic” pathway of apoptosis, the balance of pro- and anti-apoptotic Bcl-2 family proteins determines cytochrome c release from mitochondria and activation of the “initiator” caspase-9, which in turn activates the “executor” caspasess-3 and -7. In the “death receptor” or “extrinsic” pathway of apoptosis, the binding of a ligand to its death receptor activates the “initiator” caspase-8, which in turn activates the “executor” caspases-3 and -7. Compensatory activation of caspase-7 has been reported when caspase-3 is deficient or absent.
ney has no effect of on Bcl-2 or Bax protein expression in whole kidney.

There is much evidence that apoptosis is abnormally persistent in PKD and may result in cyst formation. Tubular cell apoptosis occurs in most animal models of PKD and in kidneys from humans with PKD. Mice overexpressing the proto-oncogene c-myc (SBM mice), lacking the transcription factor AP-2 beta, and Bcl-2 deficient mice have increased apoptosis and develop cysts in the kidney. Apoptosis is essential for MDCK cell cyst cavitation in collagen type I matrix and cystogenesis in this system is inhibited by overexpression of the anti-apoptotic gene, Bcl-2. 4) Expression of caspase-3 in MDCK cells results in tubule formation, whereas control cells undergo apoptosis and form cysts. A direct cause-and-effect relationship between apoptosis and cyst formation is demonstrated by our previous study in a rat model of PKD that caspase and apoptosis inhibition with IND-8050 decreases renal weights and kidney weights were the same as aged-matched wild-types. In this regard, Kuida et al. also described that the kidneys of young caspase-3−/− mice were histologically normal.

cpk+ mice in the C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME). The cpk mouse is a model of ARPKD. Thus, heterozygous cpk mice (cpkl/) do not have PKD, whereas homozygous cpk mice develop PKD. In the manuscript, the term “cpk” is used to refer to homozygous mice carrying 2 copies of the cpk gene (cystic cpk/cpk mice). The study protocol was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee. Mice had free access to tap water and standard mouse chow.

The development of the double-knockout of both the caspase-3 and cpk genes was a prolonged and complex 2-step process. Caspase-3−/− mice are not good breeders. The cpk/cpk mice do not breed because they die of PKD at about 32 d of age. In step 1, we crossed cpk heterozygous (cpkl/) mice with caspase-3 deletion heterozygous mice (casp3−/+). Statistically, one in 4 of the offspring would be cpk heterozygous and caspase-3 deletion heterozygous mice (caspkl/casp3−/+). In step 2, the (caspkl/casp3−/+ /caspkl/casp3−/+ ) mice were used as breeding pairs depending on the gender that was not always in equal numbers. The chance of the cpkl/casp3−/+ /caspkl/casp3−/+ pairs producing a caspase-3 homozygous deletion cpk homozygous mouse (double-knockout mouse) is 1 in 16. The number of double-knockout mice and littermate controls developed in the more than 4 yr duration of the study was: cpk littermate controls (n = 6), cpk/casp3−/+ (n = 5), and cpk/casp3−/− mice (n = 3).

The double-knockout mice were sacrificed when they were looking sick and were expected to die within the next 24 h. The mice were killed at that time to obtain blood and viable tissue for examination.

Genotyping of cpk/casp3−/+ and cpk/casp3−/− mice Genotyping of the offspring was performed by polymerase chain reaction (PCR) of tail DNA extracts. The cpk gene encodes a 145 amino acid protein termed Cystin. In cpk mouse, there is a tandem deletion of 12-bp and 19-bp in exon 1 of the cpk gene. Therefore, the cpk mutations can be identified using a PCR primer set flanking the deletions. The following exon1 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 TCT CCT ATC TCT CCA-3′; 3′ CPK: 5′-ATC CAG CAG GCG TAG GGT CTG-3′. The PCR condition was as follows: 95°C for 2 min, then 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min.

Caspase-3 gene-deficient mice were generated by homologous recombination, which replaced part of caspase-3 gene with a neomycin resistance gene. Caspase-3−/− mice were then bred on a C57BL/6 background. PCR genotyping of wild-type and deficient caspase-3 was performed using three primers, the sense primer for the wild-type allele 5′-CPP32, 5′GGG AAA CCA ACA GTA GTC AGT CCT-3′; the sense primer for the mutant allele derived from the neomycin cassette.

CONCISE METHODS

Generation of cpk/casp3−/+ and cpk/casp3−/− mice Caspase-3−/− mice in the C57BL/6 background were obtained from Richard A. Flavell, Howard Hughes Medical Institute, Yale University School of Medicine. The caspase-3−/− mice were originally bred on a B6/129 background.22 The C57BL/6 caspase-3−/− mice used in the present study were obtained from litters that were originally backcrossed to C57BL/6 for more than 10 generations, ensuring a uniform genetic background. In addition, the caspase-deficient mice were crossed with cpk mice (in the pure C57BL/6 background) at least an additional 5 times before double-knockout mice were obtained.

We confirmed that the kidneys of the C57BL/6 caspase-3−/− mice used in the present study were normal. The kidneys of 130-d-old caspase-3−/− mice were histologically normal. The renal function of caspase-3−/− mice as determined by BUN and serum creatinine was normal and not different from wild-type controls. The total body weights and kidney weights were the same as aged-matched wild-types. In this regard, Kuida et al. also described that the kidneys of young caspase-3−/− mice were histologically normal.

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METPHIL, 5’-TGC TAA AGC GCA TGC AGA CTG AAA TTC CPP-3’; and the antisense primer 3’-CPP32, 5’-GCG AGT GAG AAT GTG CAT AAA TTT CTP-3’. The primer sets amplified a 320 bp DNA fragment for wild-type caspase-3 and a 300 bp DNA fragment for deficient caspase-3. The PCR condition was as follows: 33 cycles of 96°C for 45 s, 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and then one cycle of 72°C for 5 min.

Western Blotting
Kidney tissue was homogenized in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) plus protease inhibitor cocktail (p-8340, Sigma-Aldrich, St Louis, MO). Equal amounts of protein (100 μg per lane) were separated on 12% SDS-PAGE and then transferred to Immobilon-p membrane (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat dry milk in TBST buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 2 h. The membranes were incubated with the following primary antibodies for 2 h at room temperature: 1) a caspase-3 antibody (sc-7148, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) that detects the full-length caspase-3 (32 kDa); 2) a caspase-7 antibody (1:1000, Cell Signaling Technology, Danvers, MA, catalog no. 9492) that detects the full-length caspase-7 (35 kDa); 3) a Bcl-2 antibody (sc 492, 1:1000, Santa Cruz Biotechnology) that detects a peptide mapping at the N-terminus of Bcl-2 of human origin (26 kDa); and 4) a Bax antibody (sc 493, 1:1000, Santa Cruz Biotechnology) that detects the a peptide mapping at the N-terminus of Bax of human origin (23 kDa). Membranes were washed in TBST buffer and further incubated with goat anti-rabbit IgG-HRP secondary antibody (sc 2030, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) that detects the a peptide mapping at the N-terminus of Bax of human origin (23 kDa). Equal protein loading (100 μg per lane) was confirmed by Coomassie Blue staining of membranes.

Quantitation of TUNEL positive cells
The TUNEL method was used to detect in situ DNA strand breaks. TACS 2 TdT-blue label in situ apoptosis detection kit (Trevenig, Gaithersburg, MD) was used. Briefly, the tissue sections were incubated with proteinase K for 1 h at 37°C for permeabilization. Endogenous peroxidase activity was quenched by incubating the tissue sections with 3% hydrogen peroxide in methanol for 5 min. The sections were then incubated with labeling reaction mix for 1 h at 37°C. After labeling with streptavidin-horseradish peroxidase, the apoptotic cells were detected with Blue Label. The number of apoptotic cells per tubule was counted by using a Nikon Eclipse E400 microscope equipped with a digital camera connected to SPOT ADVANCED 3.5 imaging software by an observer blinded to the identity of the rat kidney, using point counting stereology.24 Areas of the cortex at 90°, 180°, and 270° from the hilum of each section were selected to guard against field selection variation.

Statistical Analysis
Values are expressed as mean ± SEM. Non-normally distributed data were analyzed by the nonparametric unpaired Mann-Whitney test. Multiple group comparisons were performed using a one-way analysis of variance with post test according to Newman-Keuls. A P value of <0.05 was considered statistically significant.

ACKNOWLEDGMENTS
This work was supported by NIH grants RO1-DK074835, PO1-DK-34039, and K08-DK067191.

Portions of this study were presented at the World Congress of Nephrology Meeting in 2006 and the American Society of Nephrology Meetings in 2005 and 2006.

DISCLOSURES
None.

REFERENCES


