Treatment with 2,4-Dihydroxybenzoic Acid Prevents FSGS Progression and Renal Fibrosis in Podocyte-Specific Coq6 Knockout Mice

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ABSTRACT

Background Although studies have identified >55 genes as causing steroid-resistant nephrotic syndrome (SRNS) and localized its pathogenesis to glomerular podocytes, the disease mechanisms of SRNS remain largely enigmatic. We recently reported that individuals with mutations in COQ6, a coenzyme Q (also called CoQ10, CoQ, or ubiquinone) biosynthesis pathway enzyme, develop SRNS with sensorineural deafness, and demonstrated the beneficial effect of CoQ for maintenance of kidney function.

Methods To study COQ6 function in podocytes, we generated a podocyte-specific Coq6 knockout mouse (Coq6podKO) model and a transient siRNA-based COQ6 knockdown in a human podocyte cell line. Mice were monitored for development of proteinuria and assessed for development of glomerular sclerosis. Using a podocyte migration assay, we compared motility in COQ6 knockdown human podocytes and control podocytes. We also randomly assigned 5-month-old Coq6podKO mice and controls to receive no treatment or 2,4-dihydroxybenzoic acid (2,4-diHB), an analog of a CoQ precursor molecule that is classified as a food additive by health authorities in Europe and the United States.

Results Abrogation of Coq6 in mouse podocytes caused FSGS and proteinuria (>46-fold increases in albuminuria). In vitro studies revealed an impaired podocyte migration rate in COQ6 knockdown human podocytes. Treating Coq6podKO mice or cells with 2,4-diHB prevented renal dysfunction and reversed podocyte migration rate impairment. Survival of Coq6podKO mice given 2,4diHB was comparable to that of control mice and significantly higher than that of untreated Coq6podKO mice, half of which died by 10 months of age.

Conclusions These findings reveal a potential novel treatment strategy for those cases of human nephrotic syndrome that are caused by a primary dysfunction in the CoQ10 biosynthesis pathway.

>55 monogenic causes of SRNS has revealed that podocytes are the primary cell type affected in SRNS. Podocytes are specialized epithelial cells with a complex structure that have elaborate interdigitating foot processes, which form the slit diaphragm, a filtration barrier that consists of various cytoskeletal proteins. Maintenance of podocyte structure and function requires a highly regulated amount of energy, suggesting a high sensitivity to alteration of oxidative metabolism. Gene identification has revealed that mutations in genes encoding cytoskeletal components and components of the mitochondrial enzymes cause SRNS. Coenzyme Q (CoQ), or ubiquinone, is a redox-active lipophilic molecule and a critical component of the mitochondrial inner membrane, where it functions in the electron transport chain by transferring electrons from complexes I and II to complex III. CoQ acts also in nucleotide synthesis. It displays potent antioxidant activity in its reduced form, thus protecting cellular membranes from lipid peroxidation. De novo CoQ production involves a complex but poorly understood biochemical pathway, depending on the activity of at least ten different enzymes. Recently, mutations in genes encoding CoQ biosynthesis pathway enzymes PDSS2, COQ2, COQ6, and ADCK4 have been reported to cause SRNS. Discovery of monogenic forms of SRNS that represent primary mitochondrial diseases due to deficiency in CoQ10 levels has identified a subset of patients with SRNS, who may benefit from treatment with dietary CoQ10 or its precursor analogs. In order to test this hypothesis, we generated a podocyte-specific Coq6 knockout mouse line and a transient human podocyte knockdown cell line.

**METHODS**

**Mouse Breeding and Maintenance**

The Nphs2.Cre+;Coq6loxP/loxP mouse model on C57BL/6 genetic background used in this study was generated from targeted Coq6 ES cells obtained from the Knockout Mouse Project (EUCOMM) Repository. Coq6 ES cells were injected into blastocysts to generate Coq6-transgenic mice. Coq6−/loxP mice were crossed with Nphs2.Cre+ mice and double heterozygous mice were backcrossed to generate podocyte-specific Nphs2.Cre+;Coq6loxP/loxP knockout mice and littermate controls (Supplemental Figure 1A). Genotypes of animals were assessed by PCR (Supplemental Figure 1, B and C). Genotyping primer sequences are available upon request.

**Histologic and Ultrastructural Analyses**

Kidneys were harvested immediately after euthanasia and submerged in fixative—4% paraformaldehyde for histology, or 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) for transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Kidney serial sections were subjected to staining with hematoxylin and eosin, periodic acid–Schiff, Masson’s trichrome, and Jones’ methenamine silver following standard protocols, as well as histologic analyses. TEM and SEM were carried out using standard techniques.

**Immunofluorescence Analyses**

Mice kidneys were frozen in optimal cutting temperature compound and sectioned at 5 μm. These sections were fixed with PBS containing 4% paraformaldehyde, blocked with PBS containing 10% donkey serum and 1% BSA for 90 minutes at room temperature, then incubated with primary antibody at 4°C overnight. Sections were washed several times with PBS and incubated with fluorophore-conjugated secondary antibodies (Invitrogen) for 45 minutes at room temperature. A Leica SP5X confocal microscope was used for imaging. Image processing was done using Leica AF, ImageJ, and Adobe Photoshop CS6 software.

**Isolation and Characterization of Mouse Glomeruli**

Mouse glomeruli were isolated as described previously in a modified fashion due to perfusion. Briefly, kidneys were harvested immediately after euthanasia and perfused via renal artery with HBSS containing Dynabeads M-450, digested in Collagenase 1A and DNase I solution, glomeruli were separated on DynaMag-2 magnet and glass-glass homogenized in...
Pierce RIPA lysis buffer containing Halt Protease Inhibitor and Halt Phosphatase Inhibitor cocktails. Protein concentration was determined by DC Protein assay (Bio-Rad). An equal amount of protein was subjected to SDS–PAGE followed by western blotting. Band intensity was assessed with Image Lab software.

**Mouse Drinking Water Supplementation with 2,4-Dihydroxybenzoic Acid**

2,4-Dihydroxybenzoic acid (2,4-diHB) was administered to the mice in the drinking water at 25-mM concentration and changed twice a week. The treatment was started at 5 months of age and continued up to 10.5 months of age.

**Cell Lines and Cell Culture**

Experiments shown in this publication were performed in HEK293T cells and immortalized human podocytes. HEK293T cells were purchased from the ATCC biologic resource center. Human immortalized podocytes were a kind gift from Moin Saleem, University of Bristol, Bristol, UK, and were cultured as previously described. HEK293T cells were maintained in DMEM supplemented with 10% FBS, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Podocytes were maintained in RPMI plus GlutaMAX-I (Gibco) supplemented with 10% FBS, 50 IU/ml penicillin/50 μg/ml streptomycin, and 1% insulin-transferrin-selenium-X. Cell lines were tested for mycoplasma contamination on a biweekly basis.

**RNAi Knockdown in Human Podocyte Cell Line**

To achieve a transient knockdown human, podocytes were transfected with siRNA using RNAiMAX per manufacturer instructions. Immortalized human podocytes were transfected either with scrambled siRNA or with siRNAs targeting human COQ6. Thirty-six hours after transfection, 4×10⁴ cells were seeded in serum-free medium in the upper chambers of the migration plate. The lower chambers were filled with medium containing 10% FBS as chemo attractant, or with serum-free medium as control. Indicated supplements were added to the media in the lower and upper chambers before the assay was started. Changes in impedance were analyzed using the RTCA software. Results were plotted as cell index (relative podocyte migration) versus time.

**Isolation of Mitochondria**

Mitochondria from HEK293T and human podocyte cell lines were isolated using a mitochondria isolation kit (ThermoFisher) per manufacturer instructions. Isolated mitochondria were lysed in Pierce RIPA lysis buffer containing Halt Protease Inhibitor and Halt Phosphatase Inhibitor cocktails. Equal amounts of isolated mitochondria were subjected to SDS–PAGE and western blotting. Band intensity was assessed with Image Lab software.

**Podocyte Migration Assays**

The podocyte migration assay using xCELLigence system was performed as previously described. Briefly, real-time migration assays were performed using the xCELLigence system (Roche Applied Science) with CIM-plate 16 per manufacturer instructions. Statistic Analyses

Statistical analyses were performed using Graph Pad Prism 7 software. Significance was determined at P<0.05. Particular tests performed in the experiments are indicated in the figure legends.

**RESULTS**

**Podocyte-Specific Knockout of Coq6 Leads to Increased Mortality in Adult Mice**

To study the role of Coq6 in normal kidney function, we generated a transgenic Coq6 (Coq6<sup>tm1a</sup>) mouse line using embryonic stem cells from EUCOMM (Supplemental Figure 1A). Proper targeting of the Coq6 gene was confirmed by gene-specific genotyping (Supplemental Figure 1B). Whole body loss of Coq6 in Coq6<sup>tm1a</sup> mice was found by us to be not compatible with life. To circumvent Coq6<sup>tm1a</sup> embryonic lethality, we generated podocyte-specific Coq6 knockout mice Nphs2<sup>Cre<sup>+</sup>;Coq6<sup>lox/+</sup></sup> (hereafter referred to as Coq6<sup>podKO</sup>), by
Figure 1. Nphs2.Cre<sup>+</sup>;Coq6<sup>flo</sup>/<sup>flo</sup> mice develop severe progressive proteinuria and consecutive death in adulthood. Treatment with 2,4-diHB prevents disease progression, resulting in normal survival range. (A) Nphs2.Cre<sup>+</sup>;Coq6<sup>flo</sup>/<sup>flo</sup> mutant mice exhibit reduced life span with a median survival of 306 days, whereas Nphs2.Cre<sup>+</sup>;Coq6<sup>flo</sup>/<sup>flo</sup> mutant mice under treatment with 2,4-diHB have similar survival to healthy and healthy treated littermate controls. Dotted line displays onset of renal failure. n=10–12 animals in each group. Log-rank (Mantel–Cox) test, P<0.001. (B) Urinary albumin-to-creatinine ratio serial analysis at indicated ages and genotypes (n=10–12 animals in each group) reveals progressive proteinuria.
crossing Podocin-Cre mice with Coq6<sup>fl</sup>x<sup>lox</sup> mice in which two loxP sites surround exon 6 in the Coq6 gene. We confirmed by PCR that Podocin-Cre–dependent Coq6 inactivation occurs only in the kidneys, and not in other organs (Supplemental Figure 1C). Successful deletion of Coq6 expression in podocytes was confirmed by western blotting of glomerular lysates from Coq6<sup>podKO</sup> kidneys, where Coq6 protein levels were reduced compared with Podocin-Cre<sup>−</sup> controls (Supplemental Figure 2A). Although Coq6<sup>podKO</sup> mice appeared grossly normal, we noticed increased morbidity (hunched posture, scruffy fur) (Supplemental Figure 3A) and increased mortality (Figure 1A) in older (>10-month-old) Coq6<sup>podKO</sup> mice, features that were not observed in littermate controls. Necropsy of 10-month-old Coq6<sup>podKO</sup> mice revealed that the mutant kidneys were pale and smaller than normal (Supplemental Figure 3B), indicating that podocyte-specific deletion of Coq6 leads to structural and functional kidney defects in Coq6<sup>podKO</sup> mice.

**Coq6<sup>podKO</sup> Mice Develop Progressive Glomerular Sclerosis and Proteinuria**

To characterize the progression of kidney functional decline we followed Coq6<sup>podKO</sup> mice by monthly urinalysis for 10 months (Figure 1B). The first significant increase in the albumin-to-creatinine ratio (7.4-fold, P<0.04) in Coq6<sup>podKO</sup> mice was observed at 5 months of age (Supplemental Figure 2B). The onset of kidney functional decline in Coq6<sup>podKO</sup> mice was associated with mild focal glomerular sclerosis and occasional protein casts in the proximal tubule (Supplemental Figure 2C). Staining with Jones’ methenamine silver stain did not reveal drastic alterations in the glomerular basement membrane at this stage (Supplemental Figure 2C). However, kidney histologic analysis in 10-month-old Coq6<sup>podKO</sup> mice using periodic acid–Schiff and Masson’s trichrome staining revealed progressive and more pronounced focal glomerular sclerosis, obliteration of capillary lumens, and thickening of the basement membrane (Figure 1C). In addition, Masson’s trichrome staining revealed extensive tubulointerstitial fibrosis in the mutant kidneys (Figure 1C). Interestingly, male Coq6<sup>podKO</sup> mice were more susceptible to proteinuria compared with female mice. The increase over time in albuminuria was up to 46.9-fold in Coq6<sup>podKO</sup> mice compared with littermate controls (Figure 1B).

Recently, 2,4-diHB, a metabolic intermediate of CoQ10, has been successfully applied to ameliorate disparate phenotypes in mouse models caused by heterogeneous enzymatic defects in the CoQ<sub>10</sub> biosynthesis pathway. Given that renal structural abnormalities and functional decline manifest only relatively late in Coq6<sup>podKO</sup> mice, we decided to initiate the treatment in 5-month-old mice. Mice were randomly assigned to study groups. Administration of 2,4-diHB led to significantly increased survival of Coq6<sup>podKO</sup> mice compared with untreated Coq6<sup>podKO</sup> mice (P<0.001), who presented with 50% mortality by 10 months of age (Figure 1A). Decline in mortality was associated with significantly improved kidney function and normal glomerular histology in 10-month-old treated Coq6<sup>podKO</sup> mice (Figure 1, B and C, Supplemental Figure 4). Supplementing 2,4-diHB did not have any effect on control mouse kidney function or histology (Figure 1, B and C, Supplemental Figure 4) and ameliorated the physical condition of Coq6<sup>podKO</sup> mice (Supplemental Figure 3C), revealing normal appearance of kidneys in Coq6<sup>podKO</sup> mice after necropsy (Supplemental Figure 3D). To characterize the glomerular phenotype in Coq6<sup>podKO</sup> mice we quantified the number of sclerotic glomeruli in the treated and nontreated mutant mice and found that, although nontreated Coq6<sup>podKO</sup> mice had significantly increased numbers of sclerotic glomeruli (93.87%), treatment with 2,4-diHB effectively mitigated the sclerotic phenotype in Coq6<sup>podKO</sup> (4.95%) kidneys (Figure 2).

**Loss of Coq6 Leads to Podocyte Foot Process Effacement**

To characterize the architectural changes in Coq6<sup>podKO</sup> glomeruli at the ultrastructural level we performed TEM and SEM studies in 5-month-old and 10-month-old Coq6<sup>podKO</sup> kidneys. Changes in Coq6<sup>podKO</sup> mouse glomeruli were first observed in 5-month-old mice, with TEM revealing podocyte foot process effacement and SEM demonstrating simplified morphology of the podocytes in Coq6<sup>podKO</sup> glomeruli (Supplemental Figure 5, A and B). The podocyte architecture became progressively more abnormal in Coq6<sup>podKO</sup> kidneys by 10 months of age, characterized by lack of primary and foot processes, whereas treatment with 2,4-diHB helped to maintain the normal podocyte configuration in Coq6<sup>podKO</sup> glomeruli (Figures 3A and 4). To characterize the architectural changes in Coq6<sup>podKO</sup> glomeruli we compared the number of filtration slit units per micrometer of basement membrane in Coq6<sup>podKO</sup> and wild-type glomeruli. Whereas filtration slit frequency was significantly diminished in Coq6<sup>podKO</sup> mice, treatment with 2,4-diHB preserved the normal filtration slit.

in Nphs2.Cre<sup>+</sup>;Coq6<sup>fl</sup>x<sup>lox</sup> mutant mice (red square) but not in littermate controls (black circle). Nphs2.Cre<sup>+</sup>;Coq6<sup>fl</sup>x<sup>lox</sup> mutant mice under treatment with 2,4-diHB (green triangles) are protected from developing proteinuria. Dotted line displays onset of renal failure. Note that once chronic renal failure ensues, urinary albumin excretion is reduced as is known to occur in SRNS. One-way ANOVA under treatment with 2,4-diHB preserved the normal glomerular architecture in 10-month-old Coq6<sup>podKO</sup> mice (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; each data point represents mean value of technical duplicates, error bars represent SEM. (C) Kidney serial sections and representative images of 10.5-month-old mice with indicated genotypes were stained according to indicated conditions. The Nphs2.Cre<sup>+</sup>;Coq6<sup>fl</sup>x<sup>lox</sup> mutant mice exhibit FSGS (arrows) with focal interstitial fibrosis, tubular atrophy (arrow heads), and proteinaceous casts in dilated tubules (asterisks). In contrast, wild-type littermate control mice and Nphs2.Cre<sup>+</sup>;Coq6<sup>fl</sup>x<sup>lox</sup> mutant mice under treatment with 2,4-diHB display normal histologic kidney morphology. Scale bars, upper rows 500 μm and lower rows 20 μm. PAS, periodic acid–Schiff.
morbidity in Coq6podKO glomeruli (Figure 3B). Overall, the glomerular phenotype of podocyte-specific loss of Coq6 recapitulates the human pathology of FSGS of COQ6-deficient individuals.21

**Abrogation of Coq6 Expression Leads to Podocytopathy**

To characterize the molecular abnormalities in Coq6podKO glomeruli, we next analyzed the expression patterns of the slit diaphragm proteins nephrin and podocin, the basement membrane marker nidogen, and the primary process marker synaptopodin by confocal microscopy in 10.5-month-old kidneys (Figure 5). A staining pattern for various podocyte markers was significantly reduced in Coq6podKO glomeruli (Figure 5, A and C, Supplemental Figure 6), whereas the basement membrane marker nidogen showed a wider expression than normal, demonstrating that Coq6 function is required for podocyte maintenance and homeostasis. Because glomerular sclerosis is associated with increased expression of fibrotic markers, we next analyzed the expression of fibrotic markers αSMA, collagen IV, and Desmin in Coq6podKO kidneys by confocal microscopy. Indeed, Coq6podKO kidneys had significantly increased expression of αSMA, Desmin, and collagen IV in the glomeruli, characteristic of mesangial fibrosis (Supplemental Figures 7–9). We also studied the expression of WT1, a podocyte-specific transcription factor, which is critical for maintaining podocyte differentiation and maturation.36,37 In contrast to control glomeruli, the number of WT1+ podocytes was reduced in Coq6podKO glomeruli, suggesting that loss of Coq6 expression either leads to progressive depletion of podocytes (Supplemental Figure 10) or causes their de differentiation. Together, our data show that treatment of Coq6podKO mice with 2,4-diHB protects from disease progression and ameliorates the outcome regarding renal histology.

**Mitochondrial Deficiency Underlies the Glomerular Abnormalities in Coq6podKO Mice**

Although Coq6 function is associated with the mitochondrial CoQ10 biosynthesis pathway, the subcellular localization of Coq6 has remained controversial.21 Using cellular fractionation of HEK293 and human podocyte cell lysates, we now demonstrate that endogenous Coq6 localizes to the mitochondria (Figure 6, A and B), consistent with Coq6's localization and function in the mitochondria. The podocytes in Coq6podKO glomeruli appeared to contain abnormal mitochondria (characterized by hyperproliferation and increased size), presumably to compensate for defective energy metabolism (Figure 6C, Supplemental Figure 11).

**Figure 2.** Development of FSGS is abrogated by treatment with 2,4-diHB in Nphs2.Cre+;Coq6fl/fl mutant mice. Kidney serial sections of 10.5-month-old mice with indicated genotypes were stained with Masson’s trichrome and analyzed to determine the severity of glomerular sclerosis. The Nphs2.Cre+;Coq6fl/fl mutant mice show FSGS, whereas Nphs2.Cre+;Coq6fl/fl mutant mice under treatment with 2,4-diHB displayed virtually no (<10% of glomeruli) abnormal histologic findings (n=3 animals in each group; each graph bar indicates an single animal, numbers inside the bar graphs indicate number of sclerosed glomeruli per total glomeruli counted in one section. Two-way ANOVA P values calculated using Tukey’s multiple comparisons test are shown in the figure. ****P<0.001.
To further characterize the role of COQ6 in podocyte function, we employed COQ6 knockdown human podocytes in a cellular migration assay that we recently established to identify compounds that modulate podocyte mobility.38 Using transient, siRNA-based COQ6 knockdown in human podocytes (Supplemental Figure 12), we observed reduced migration rate in COQ6-depleted cells compared with control podocytes (Supplemental Figure 12B), emphasizing the role of COQ6 in normal podocyte function. Supplementing 2,4-diHB to the culture medium mitigated the migration defect (Supplemental Figure 12B). Having shown that the defect in podocyte migration rate is a functional and specific readout of COQ6 deficiency, we decided to use this cellular model to test the effects of two other 4-dihydroxybenzoic acid (4-HB) analogs, 4-Hydroxy-3-methoxybenzoic acid (vanillic acid) and 3,4-Dihydroxybenzoic acid (3,4-diHB), in rescuing COQ6 deficiency. Similar to 2,4-diHB, these compounds are all highly similar to 4-HB, a precursor molecule of CoQ10 biosynthesis. Previously, it has been demonstrated that treatment with vanillic acid or 3,4-diHB improved the biosynthesis of COQ6 in a yeast model.39,40 Indeed, using vanillic acid we saw a partial reversal of the migratory defect in COQ6 knockdown cells (Supplemental Figure 12C); however, treatment with 3,4-diHB showed no positive effect to the siCOQ6 podocyte migration phenotype (Supplemental Figure 12D). Together, these data demonstrate that loss of COQ6 function in podocytes can be partially reversed by vanillic acid and fully reversed by 2,4-diHB of the tested podocyte.

Figure 3. Glomerular structure is disrupted in Nphs2.Cre⁺;Coq6floxflox mutant mice and is preserved by 2,4-diHB treatment. (A) TEM representative images at age 10.5 months. Nphs2.Cre⁺;Coq6floxflox mutant mice reveal podocyte foot process effacement (arrows) and an increased number of mitochondria (asterisks). Glomerular basement membrane is highlighted by a dotted line. In contrast, Nphs2.Cre⁺;Coq6floxflox 2,4-diHB–treated mice display normal foot process morphology. Scale bars, 2 μm left panel, 1 μm middle and right panels. (B) TEM images of 10.5-month-old mice with indicated genotypes (see A) were analyzed to determine the severity of podocyte foot process effacement. The Nphs2.Cre⁺;Coq6floxflox mutant mice show significantly reduced frequency of filtration slits per micron of glomerular basement membrane, whereas Nphs2.Cre⁺;Coq6floxflox 2,4-diHB–treated mice under treatment with 2,4-diHB display normal ultrastructural findings compared with littermate controls under treatment. n=2 animals in each group; two glomeruli per animal were analyzed. One-way ANOVA P values calculated using Tukey’s multiple comparisons test are shown in the figure. ****P<0.0001; error bars represent mean±SD. GBM, glomerular basement membrane.
CoQ\textsubscript{10} intermediate precursors, at least under \textit{in vitro} conditions.

**DISCUSSION**

The CoQ\textsubscript{10} biosynthesis pathway is thought to consist of sequential enzymatic steps\textsuperscript{41}. Moreover, studies in yeast have demonstrated that CoQ biosynthesis pathway enzymes assemble into a high molecular mass protein complexes, whose stability is dependent on the presence of individual peptides\textsuperscript{42,43}. There is a wide spectrum of clinical symptoms, including neurologic disorders, myopathy, and SRNS, in patients caused by deficiency of CoQ\textsubscript{10} in consequence of mutations in a variety of genes involved in the CoQ\textsubscript{10} biosynthesis process\textsuperscript{18,20–23,29,44–50}. On the basis of these data, it is very important to accurately identify these treatable patients as early as possible.

Several recent \textit{in vitro} and \textit{in vivo} studies, including ours, have demonstrated the beneficial effect of CoQ\textsubscript{10} in improving the outcome of primary mitochondrial diseases due to CoQ\textsubscript{10} deficiency\textsuperscript{51,52}. Because CoQ\textsubscript{10} requires high-dose use and is not water soluble, limiting its use in cell culture systems, we decided to examine the effect of its more soluble and hydrophilic 4-HB analogs—2,4-diHB, 3,4-diHB, and vanillic acid—on podocyte function.

There are controversial data in the literature regarding the efficacy of using oral CoQ\textsubscript{10} in patients with primary CoQ\textsubscript{10}...
mitochondriopathy, likely stemming from CoQ10 tissue-specific bioavailability, its limited delivery to mitochondria, and gene-specific mutations. For example, previous studies have demonstrated the beneficial effect of oral CoQ10 for neural improvements, and in some cases partially for renal dysfunction. After we had demonstrated the beneficial effect of CoQ10 precursor compounds for podocyte functional improvement by migration assay we decided to investigate whether the compounds can attenuate proteinuria in Coq6podKO mice. On the basis of our data and data in the literature we selected 2,4-diHB to answer this question.

Coq6podKO mice appeared to be normal in development and body condition. Upon the onset of significant proteinuria at age 5 months, Coq6podKO mice appeared to deteriorate gradually and became moribund at 10 months of age with advanced decline of renal function. Histologic analysis of 10-month-old Coq6podKO mice revealed progressive FSGS associated with simplified morphology of podocytes and foot process effacement. Immunofluorescence studies showed decreased expression of podocyte markers and increased expression of fibrotic markers. Coq6podKO mice treated with 2,4-diHB were protected from disease progression, and survival, proteinuria, and renal histology improved dramatically. Studies in cultured human podocytes with transient knockdown of COQ6 showed reduction in podocyte migration rate that could be completely reversed to control levels by treating the podocytes with 2,4-diHB.

Together, our data demonstrate that 2,4-diHB, an analog of 4-HB that functions to bypass certain deficiencies of the CoQ10 biosynthesis pathway, efficiently ameliorates proteinuria and prevents FSGS in Coq6podKO mice. There was a sex-specific susceptibility to proteinuria in Coq6podKO mice, with female mice being more resistant, and male mice more susceptible to proteinuria after deletion of Coq6.

Figure 5. Nphs2.Cre⁺;Coq6flox/flox mutant mice show reduced expression of podocyte-specific proteins. (A and B) Immunofluorescence staining of frozen kidney sections and representative images in 10.5-month-old mice for the slit diaphragm proteins (green) (A) podocin and (B) nephrin and the basement membrane marker nidogen (red). A normal expression pattern of podocin and nephrin is seen in wild-type littermate control mice. Nphs2.Cre⁺;Coq6flox/flox mutant mice show (A) reduced podocin staining (arrows) appearing only on a few capillary loops (arrow head), whereas (B) nephrin expression is globally reduced. Scale bars, 10 μm. (C) Staining of frozen kidney sections and representative images in 10.5-month-old mice for the podocytic foot process marker synaptopodin (green) and the basement membrane marker nidogen (red). A normal expression pattern of synaptopodin is seen in wild-type littermate control mice. Nphs2.Cre⁺;Coq6flox/flox mutant mice show reduced synaptopodin (green) staining (arrows) with a signal appearing only on a few capillary loops (arrow head). Scale bars, 10 μm.
Figure 6. COQ6 localizes to mitochondria and its loss from glomerular podocytes causes mitochondrial intumescence. (A) Subcellular fractions of wild-type HEK293T cell line and of wild-type undifferentiated human podocyte cell line were subjected to western blot analysis. COQ6 was detected in both cell lines, predominantly in the mitochondrial fraction. CoxIV and α-Tubulin were used as markers for mitochondrial and cytosolic subcellular fractions, respectively. Representative image of two independent experiments. (B) Mitochondrial fraction does not contain Golgi complex proteins. Whole cell lysates and the mitochondrial fraction from HEK293T and human podocytes were analyzed using antibodies against the Golgi complex protein 58K, mitochondrial protein CoxIV, and COQ6. 58K was not detected in the mitochondrial fraction, confirming that COQ6 is a mitochondrial protein. (C) TEM of podocytes in 10.5-month-old mice. Compared with the number and morphology of mitochondria in wild-type podocytes, the mitochondria in Nphs2.Cre<sup>+</sup>;Coq6<sup>fl/flox</sup> podocytes are enlarged and increased in number indicating impairment of mitochondrial function. Scale bars, 1 μm. Undif, undifferentiated.
reason for this is unclear. However, several aspects of siCOQ6 podocyte physiology were rescued with 4-HB analogs, with 2,4-diHB having the most robust effect. It is surprising that 2,4-diHB was most effective in the Coq6pod/KO model of SRNS caused by primary mitochondrial dysfunction. This effect is elicited without the risk of adverse effects, because several “hydroxybenzoic acid compounds” and especially 2,4-diHB are FDA- (EAF 3045, CAS RN 89–86–1) and EFSA- (Ref. no. 00910) approved food additives. In addition, 2,4-diHB is widely present as a by-product of food processing in a variety of food products, such as snack foods, beverages, and fish products, as well as being used as an additive in some drugs, e.g., Nicardipine (NDC: 0143–9542–01). Moreover, animal studies revealed high safety of use for 2,4-diHB with LD50 of >800 mg/kg body wt, and there is one case report of 2,4-diHB administration to a patient with rheumatic fever in a dose of up to 6 g per day without observation of any severe side effects of treatment. Because of the apparent safety in preclinical and clinical use, it is very likely that patients would very well tolerate treatment with 2,4-diHB. The translational relevance of this discovery is heightened by the feasibility of therapeutic delivery. Translational studies are warranted to determine whether this type of strategy may be used to promote proteinuria remission. It would be useful to perform animal studies with a human mutation using a knock-in strategy. Growing evidence suggests that impaired mitochondrial function causes podocyte damage and leads to proteinuria. Given the present lack of effective therapies of this disease entity and the apparent safety of CoQ10, clinical trials of these compounds in genetically identified cases of SRNS seem appropriate.

ACKNOWLEDGMENTS

The authors thank Maria Ericsson, Louis Trakimas, Elizabeth Benecchi, and Peg Coughlin from the Electron Microscope Core Facility, Harvard Medical School, for excellent transmission electron microscopy services. We also thank Evelyn Flynn for her outstanding technical assistance and Eliso Nudelman for providing mouse cartoon illustrations. This work was performed in part at the Center for Nanoscale Systems (CNS), a member of the National Nanotechnology Coordinated Infrastructure Network, which is supported by the National Science Foundation (NSF) under NSF award no. 1541959. CNS is part of Harvard University.

This study was supported by National Institutes of Health grants to E.H. (DK076683) and R.A. (R00DK099434 and R01DK115403), and by the National Science Foundation Grant MCB-1330803 (to C.F.C.). E.H. is the William E. Harmon Professor. E.W. is supported by the Leopoldina Fellowship Program, German National Academy of Sciences Leopoldina (LPDS 2015-07).

E.W., M.A., H.H., D.S., J.W., C.C.G., R.S., J.C., and M.S. carried out the animal experiments. E.W., M.A., A.M.A., A.N., C.F.C., and R.A. directed the project. E.W. and R.A. wrote the paper with help from F.H. The manuscript was critically reviewed by all of the authors.

DISCLOSURES

F.H. is a cofounder of Goldfinch-Bio. No other authors have competing financial interests.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2018060625/-/DCSupplemental.

Supplemental Figure 1. Generation and genotyping strategy for Nphs2.Cre+;Coq6lox/lox mouse model.

Supplemental Figure 2. At 5 months of age Nphs2.Cre+;Coq6lox/lox mice exhibit proteinuria and renal histopathologic changes consistent with FSGS.

Supplemental Figure 3. Coq6 knockout in Nphs2.Cre+;Coq6lox/lox mutant mice leads to reduced physical condition and macroscopic morphologic changes of kidneys.

Supplemental Figure 4. Coq6 knockout in Nphs2.Cre+;Coq6lox/lox mutant mice causes FSGS. Treatment with 2,4-diHB prevents disease progression, resulting in normal histologic findings.

Supplemental Figure 5. Electron microscopy reveals podocyte foot process effacement in Nphs2.Cre+;Coq6lox/lox mutant mice at 5 months of age.

Supplemental Figure 6. Quantitative analysis of the expression of podocyte-specific proteins in Nphs2.Cre+;Coq6lox/lox glomeruli.

Supplemental Figure 7. Nphs2.Cre+;Coq6lox/lox mice show increased glomerular fibrosis and staining for mesangial markers.

Supplemental Figure 8. Quantitative analysis of the expression of the fibrotic markers aSMA and Desmin in Nphs2.Cre+;Coq6lox/lox glomeruli.

Supplemental Figure 9. Nphs2.Cre+;Coq6lox/lox mice develop renal fibrosis.

Supplemental Figure 10. Nphs2.Cre+;Coq6lox/lox mice show a reduced staining of podocyte-specific markers.

Supplemental Figure 11. Deletion of Coq6 leads to morphologic abnormalities in podocyte mitochondria.

Supplemental Figure 12. Coq6 siRNA-mediated transient knockdown reduces podocyte migration rate of cultured human podocytes with full rescue by 2,4-diHB, partial rescue by vanillic acid, and absent rescue by 3,4-diHB.

REFERENCES


**AFFILIATIONS**

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