Proximal tubular cell-specific ablation of carnitine acetyl-transferase causes tubular disease and secondary glomerulosclerosis.

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Abstract

Proximal tubular epithelial cells are highly energy demanding. Their energy need is covered mostly from mitochondrial fatty acid oxidation. It is suggested, but not entirely clear whether derailments in fatty acid metabolism and mitochondrial dysfunction are forerunners of tubular damage. Here we modeled mitochondrial overload by creating mice lacking the enzyme carnitine acetyl-transferase (CrAT) in the proximal tubules, thus limiting a primary mechanism to export carbons under conditions of substrate excess. Mice developed tubular disease and interestingly, secondary glomerulosclerosis. This was accompanied by increased levels of apoptosis regulator and fibrosis markers, increased oxidative stress and abnormal profiles of acylcarnitines and organic acids suggesting profound impairments in all major forms of nutrient metabolism. When mice with CrAT deletion were placed on a high fat diet, kidney disease was more severe and developed faster. Primary proximal tubular cells isolated from the knockout mice displayed energy deficit and impaired respiration before the onset of pathology, suggesting mitochondrial respiratory abnormalities as a potential underlying mechanism. Our findings support the hypothesis that derailments of mitochondrial energy metabolism may be causative to chronic kidney disease. Our results also suggest that tubular injury may be a primary event followed by secondary glomerulosclerosis, raising the possibility that focusing on normalizing tubular cell mitochondrial function and energy balance could be an important preventative strategy.
INTRODUCTION

While there is no doubt that glomerular disease is a major feature in the development of both diabetic nephropathy (DN) and obesity-related chronic kidney disease (CKD), evidence now also indicates proximal tubular injury as an important early event in kidney disease (1-3). Proximal tubular epithelial cells (PTC) are highly energy demanding and contain a large number of mitochondria. Their high energy need is primarily covered from mitochondrial fatty acid oxidation to produce ATP (4-6). While excess fatty acid metabolites can be shuttled to cytoplasmic lipid droplets to shield from toxicity, this buffer capacity is limited in PTC (7; 8). Therefore, disturbances in fatty acid oxidation appear to cause PTC atrophy through apoptosis (9-15). In type 2 diabetes and obesity, persistently elevated triglyceride and free fatty acid levels in the circulation (16) are damaging to tubular cells (17; 18). Recent discoveries show that metabolic alterations in tissues affected by diabetic complications are tissue-specific, with increased fatty acid metabolism in the kidney of type 2 diabetic mice and humans (19). Increased β oxidation however is not matched with ATP production. The kidney cortex (~90 % tubular cells) shows accumulation of intermediate metabolic products from mitochondrial β oxidation. After an initial increase, tricarboxylic acid (TCA) cycle activity slows down in advanced kidney disease and organic acid metabolites from the TCA cycle are lost into the urine. These conditions altogether are consistent with “mitochondrial lipid overload” (20-24).

Mitochondrial overload is not a new phenomenon, but its contribution to CKD is not known. It was first described in skeletal muscle in the context of insulin resistance (22) where mitochondria are presented with substrate excess. This substrate excess overwhelms the catabolic capacity of mitochondrial β oxidation. Consequently, free CoA levels decline, as increased supply and oxidation of fatty acids leads to accumulation of excess acetyl-CoA. Mitochondrial
overload also results in increased levels of incompletely oxidized products: long- and medium-chain acylcarnitines and acyl-CoAs. Sustained elevation in incomplete fatty acid oxidation then may foster a mitochondrial microenvironment conducive to oxidant stress (25). This is because a chronic increase in substrate entry to the mitochondria produces reducing equivalents in excess of that which can be handled by the electron transport chain (ETC). This leads to increased NADH and FADH$_2$ levels. Such an increase was proposed to induce electron “backpressure” and reactive oxygen species (ROS) production.

To model the aforementioned mitochondrial substrate overload, we ablated the enzyme carnitine-acetyl transferase (CrAT) in proximal tubular epithelial cells in mice (“PT-CrAT” mice). CrAT is a mitochondrial matrix enzyme, serving as a “relief valve” (26). It facilitates the export of excess, preferably short-chain acyl-CoA products out of the mitochondria by linking them to carnitine, making these products membrane permeable (23). As acylcarnitines can traverse membranes, CrAT also plays a key role in regulating cellular/mitochondrial acetyl- and acyl-CoA balance and carbon trafficking. In the absence of CrAT, acetyl-CoA/CoA ratio increases and some partially oxidized products cannot exit the mitochondria. CrAT deletion therefore leads to the accumulation of incompletely oxidized acyl-CoA products and mitochondrial acetyl-CoA overload (22; 27).

Our goal in the present study was to understand whether such conditions can be the forerunners of tubular disease. We demonstrate that PT-CrAT mice develop not only tubular but glomerular disease as well, and a high fat diet exacerbates pathology. We also provide evidence that impaired mitochondrial respiration in overloaded PTCs precedes the onset of disease development. Thus, our data indicate that PTC-specific deletion of CrAT causes kidney disease...
and that mitochondrial overload promotes the acceleration of CKD in relation to metabolic disease.

**RESEARCH DESIGN AND METHODS**

**Animals.** Mice with targeted deletion of CrAT in PTC were generated using Cre-loxP recombination strategy (Fig. 1). Homozygous CrATloxP female mice (C57BL/6J) were bred to male \( \gamma \)-glutamyl-transferase Cre mice (Tg-(Ggt1-cre)M3Egn/J, mixed Balb/cJ/C57BL/6 background, Jackson Laboratories). Offsprings heterozygous to CrATloxP were backcrossed to the CrATloxP mice. This cross produced offsprings of which 50% were PTC-specific CrAT ablated, “PT-CrAT” mice. Littermate homozygous fl/fl, Cre-negative mice were used as controls. To verify the regions of the kidney with Cre-recombinase activity, a set of PT-CrAT female mice were bred with a “tdTomato” (B6.Cg-Gt(ROSA)26Sor<tm14(CAG-tdTomato)Hze>/J) male. Kidneys of the Cre positive offsprings were dissected at 4 weeks of age, sectioned and photographed under a LeicaDM6000 fluorescent microscope. Another set of kidneys from fl/fl control and PT-CrAT mice (n=4/genotype) were harvested. One kidney per mouse was cryosectioned and stained with an anti-active-Cre antibody (1:100, Novagen). Sections were photographed as described above. The other kidney, as well as heart, skeletal muscle and epididymal white adipose tissue (eWAT) were used for qPCR and Western blot analyses to determine CrAT mRNA expression and protein levels. CrAT activity from kidney homogenates was measured as described in (23). Genotyping was performed on tail DNA using standard PCR reagents. Mice were kept in a room with a 12/12 h light/dark cycle and had access to food and water *ad libitum*. All animal studies were performed at the AAALAC accredited facility of PBRC and approved by IACUC. Mice were randomly divided into cohorts, and kept
on chow diet (5001 Purina Rodent Chow) for 6, 12, 15, 18 and 24 months. Other cohorts were kept on low fat diet (10% kcal fat from lard, D12450B, Research Diets), high fat diet (60 % kcal fat from lard, D12492) up to 18 months. At the end of each time point, mice were placed in a metabolic chamber for a 24 hr acclimation. Urine was collected every 24 hr and the 72 hr urine was used for proteinuria analysis and mass spectrometry. Fasting blood glucose levels were measured from a drop of blood from the tail vein (OneTouch). For glucose tolerance test, mice received a single dose of 10 % glucose solution (200 µl/50 g bwt, ip.) and blood glucose levels were measured at 30, 60, 90 and 120 minutes. Kidneys were excised and halved. One kidney was fixed in 4 % paraformaldehyde and processed for paraffin embedding, the other kidney was processed in OCT media for cryocutting and immunofluorescent staining.

**Histology.** Paraffin embedded kidneys were cut into 5 µm cross-sections. Sections were mounted on charged SuperFrost slides (Fisher Sci.), and deparaffinized. Sections were stained with a) Periodic acid-Schiff (PAS) staining to evaluate glomerular size, sclerosis and proteinaceous casts; b) TriChrome staining for fibrosis and collagen deposits. At least 10 viewing areas per slide were evaluated on each section with a NanoZoomer Digital Pathology Virtual Slide Viewer.

**Immunostaining.** Deparaffinized kidney sections were mounted on SuperFrost slides, permeabilized with 0.1 % Triton-X and blocked with 1 % BSA. Antibodies were diluted in PBS and applied on kidney sections using a Pep Pen as hydrophobic barrier: rabbit anti-Collagen IV, 1:250 (Abcam), mouse anti-Vimentin, 1:250, (Sigma), cleaved caspase-3, 1:200, (Abcam), *Lotus tetragonolobus* anti-lectin conjugated with green fluorophore (488 nm) 1:200 (Vector Laboratories), and corresponding AlexaFluor 598 red anti-mouse or AlexaFluor 647 cyan anti-
rabbit secondary antibodies (1:750, ThermoFisher). Slides were coverslipped (Fluoromount G) and observed using a Leica DM6000 fluorescent microscope.

**Western blotting.** Kidney cortices were homogenized in lysis buffer, normalized for protein concentration and proteins were separated using gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane and analyzed by standard Western blotting methods using the following antibodies: rabbit anti-CrAT, 1:250, (Proteintech), mouse anti-hydroxynonenal 1:500, (R&D Biosciences), mouse anti-acetyl-lysine, 1:500, (Cytoskeleton), mouse anti-OxPhos cocktail, 1:500, (ThermoFisher) and mouse anti-β actin or tubulin as loading control when applicable, 1:5000. Secondary antibodies (anti-mouse or anti-rabbit, 1:5000, conjugated with HRP, ThermoFisher) were applied at room temperature for 1 hr. Band intensities were measured using the Image J software Gel Analysis plug in.

**Primary cell culture and mitochondrial respiration analysis.** Kidneys from younger PT-CrAT and fl/fl mice (<9 months) were harvested and placed into ice cold Krebs-Henseleit buffer for primary proximal tubular epithelial cell isolation. PTCs were isolated using a collagenase digestion/sieve/Percoll gradient centrifugation method originally described by Vinay et al (28). Cells were grown in hormonally defined DMEM media (28). Once cells reached near confluency (~day 7-8), they were plated onto 24-well XF24 SeaHorse culture plates (75,000 cells/well). Mitochondrial oxygen consumption was measured in cells respirating on 5 mM pyruvate using a SeaHorse XF24 Extracellular Flux Analyzer.

**Mitochondrial complex activities.** Complex I and complex V activity were measured from repeatedly freeze-thawed kidney cortex homogenates by following the oxidation of NADH at 340 nm. For complex I activity, the final reaction mixture contained 62.5 µM ubiquinone, 0.25 % BSA, 2 µg/ml antimycin A in 25 mM potassium phosphate buffer (pH=7.2) and the sample
equivalent to 10 µg protein. Oxidation of NADH was followed at 340 nm in the absence and presence of 10 µg/ml rotenone (3 minutes each). For complex V activity, the mixture contained the sample equivalent to 5 µg protein, 10 µl of 30 mM NADH, 50 µl of 50 mM phosphoenolpyruvic acid, 5 µl of 10 mg/ml pyruvate kinase, 10 µl of 5 mg/ml lactate dehydrogenase and 10 µl antimycin A in a total of 900 µl HEPES-Mg²⁺ buffer (pH=8.0) at 30 ºC. Baseline was recorded for 2 min then 100 µl of 25 mM ATP was added and the absorbance recorded for another 2 min. Then 10 µl of 0.2 mg/ml oligomycin was added and the absorbance was recorded again for 2 min. In both cases Vmax values were calculated from the kinetic graphs and activities were expressed as control percentage.

Mass Spectrometry. Kidney cortex, plasma and urinary levels of acylcarnitines and organic acids were measured using flow injection tandem mass spectrometry at the National University of Singapore-Duke (29; 30).

EPR Spectroscopy. A cohort of mice received the spin trap POBN (500 mg/kg ip., 45 minutes before euthanasia) to analyze lipid-derived free radical production. Kidney samples were extracted using a Folch-extraction method (31; 32). Extracts were analyzed in a quartz flat cell using an X-band EMX Plus EPR spectroscope (parameters: 3480±80 G scan width, 10⁵ receiver gain, 20 mW microwave power, time constant: 1310 ms, conversion time: 655 ms).

qPCR. Total RNA from kidney cortices was used for real-time quantitative PCR to compare gene expressions (Vim, Col4A1, Col3A1a, Fn, Bax, Casp-3) between groups using SYBR Green Master Mix as described by manufacturer (Applied Biosystems). Fold changes in gene expression were calculated using the ddCT method.

Albumin and creatinine. Urinary albumin levels from 24 hr urine samples were measured using Albuwell M albumin kit (Exocell). Creatinine levels in serum and urine were measured using a
Creatinine Companion kit (Exocell). Albuminuria was expressed as albumin/creatinine ratios (µg/mg) as per AMDCC recommendation.

Statistical Analysis. Data were expressed as mean ± SD. Statistical significances between fl/fl and PT-CrAT mice or PT-CrAT mice on LF vs PT-CrAT mice on HF diet were determined by unpaired Student’s t-test.

RESULTS

PT-CrAT Mice Develop Tubular Disease and Secondary Glomerulosclerosis. Mice with PTC-specific deletion of CrAT were created using a Cre-loxP strategy, genotyped and characterized as detailed in Fig. 1. CrAT deletion was specific to the proximal tubules (Fig. 1B-G). The first cohorts of mice were kept on chow diet and for consistent development of nephropathy (33) were aged to >12 months. Renal tissue was examined every three months. PT-CrAT mice had normal body weights (Fig. 1H), kidney weights (Fig. 1I), fasting blood glucose levels (Fig. 1J) and glucose tolerance (Fig. 1K). They developed tubular disease with protein casts, tubular dilation and fibrosis around 18-20 months of age (Fig. 2B-C). Increased levels of collagen IV, vimentin and cleaved caspase-3 were detected in PT-CrAT mice using immunofluorescent techniques (Fig. 2F-I). By this time, they also had increased serum creatinine levels and proteinuria (Fig. 2D-E). Consistently with the stainings, significantly increased expression levels of vimentin, collagen IV, Bax and caspase-3 were detected by qPCR, indicating fibrosis and activation of apoptosis regulators (Fig. 2J). Interestingly and unexpectedly, PT-CrAT mice also developed glomerulosclerosis. Within a given cohort of mice, tubular/glomerular disease and proteinuria always varied to an extent, with some mice having only mild changes while others having extensive damage. These results indicate that ablation of
CrAT in the PTC leads not only to tubular disease but to proteinuric CKD and facilitates tubular cell/mitochondrial apoptosis.

**PT-CrAT Mice Have Altered Acylcarnitine and Organic Acid Profiles.** Next, to determine the extent of mitochondrial overload, we measured the levels of acylcarnitine products in fl/fl and PT-CrAT mice plasma, kidney cortex and urine. Fig. 3A-C shows the results of a detailed MS/MS analysis of major acylcarnitine species. PT-CrAT mice displayed altered acylcarnitine profiles in the kidney cortex and urine when compared to fl/fl littermates. Notably, CrAT deficiency in the PTC led to a significant decrease in the levels of acetyl- and several other short and medium-chain acylcarnitines. This result is consistent with the hypothesis of mitochondrial overload: due to the lack of CrAT in PTC, efflux capacity is attenuated and thus short chain acylcarnitine levels are much lower in the cortex and urine. Interestingly, and also consistent with previous observations in skeletal muscle (23), many of the longer chain acylcarnitine levels were increased in the kidney cortex. Furthermore, several of the measured organic acid levels (citrate, fumarate, malate, lactate and pyruvate) were depleted in the PT-CrAT cortex (Fig. 3G). We also found an approximately 2-fold increase in urinary levels of free and total carnitine in the PT-CrAT mice, though these differences did not reach statistical significance (Fig. 3D-F). Carnitine loss to the urine suggests that secondary carnitine insufficiency could develop that may be attributable to PTC injury (34).

**Lipid-derived Free Radical Production is Increased in PT-CrAT Mice.** Retaining incompletely oxidized acylcarnitine products in the mitochondria can lead to conditions favorable for redox imbalance. This is because mitochondrial overload may produce reducing equivalents (NADH and FADH$_2$) in excess of that which can be handled by the ETC (22; 25). Such excess in turn is predicted to create an environment conducive to oxidative stress. Excess
ROS in a membrane-rich environment like the mitochondrial inner membrane then would increase the odds for lipid peroxidation (35-37). To address this scenario, we used electron spin resonance spectroscopy (ESR) and *in vivo* spin trapping to measure the levels of lipid-derived free radicals in kidney cortices. Results were also confirmed by Western blot analysis of 4-hydroxynonenal adducts. Fig. 4A shows spectra obtained from fl/fl and PT-CrAT mouse kidney cortex extracts. PT-CrAT kidney cortices display an approximately 3-fold increase in carbon-centered lipid-derived free radical production when compared to fl/fl littermates. Consistently, we found a significant increase of 4-HNE adducts in CrAT ablated cortices (Fig. 4B-C). These results confirm that mitochondrial overload may increase ROS production which then also triggers excess lipid peroxidation.

**High Fat Diet Challenge Accelerates Kidney Disease in PT-CrAT Mice.** Next, we asked whether obesity and prediabetes-induced renal injury is accelerated by mitochondrial overload. We have used high fat-diet challenge to determine whether PT-CrAT mice are more susceptible to HFD-induced renal disease. HFD feeding led to an earlier manifestation (12-15 months) of CKD in the PT-CrAT model (Fig. 5A-C). When compared to fl/fl littermates on HFD, or to PT-CrAT mice on LFD, PT-CrAT mice fed a HFD had larger glomeruli (Fig. 5D) and increased expression of fibrosis genes *Vim, Fn* and *Col3A1a* (Fig. 5E). Systemic changes such as body weights, plasma triglyceride and free fatty acid levels however, were comparable between PT-CrAT colonies, regardless of the diet (Fig. 5F-H). Thus, CrAT deletion in the PTC promotes obesity/prediabetes-related kidney disease and potentially through secondary mechanisms affects glomerular structure.

**Primary PTCs from PT-CrAT mice have energy deficit and impaired mitochondrial respiration.** To further assess mechanistic details related to mitochondrial overload, we isolated
primary PTCs from fl/fl and PT-CrAT mice. PTCs were isolated from younger mice (up to 9 months) where pathology was not yet present. When mitochondrial oxygen consumption rates (OCR) were compared in an XF24 Analyzer, cells from the PT-CrAT mice displayed significantly lower basal, ATP-linked and maximal respiration (Fig. 6A-B). These results indicate early changes in mitochondrial respiratory function, and energy deficit (lower ATP-linked respiration) in PTCs with mitochondrial overload. Importantly, as these alterations are present before pathology develops in the PT-CrAT mice, mitochondrial dysfunction and energy deficit could be a primary cause of tubular cell death later. Tissue abundance of mitochondrial ETC complexes or the amount total acetyl-lysine modified proteins were not different between groups (Fig. 6C-D) indicating similar mitochondrial content in control and CrAT ablated mice. However, complex I and complex V activities were impaired (Fig. 6E).

**DISCUSSION**

Mitochondrial overload was first described in skeletal muscle, in relation to the development of insulin resistance (22; 25). The effects of CrAT deletion were also demonstrated first in skeletal muscle, with many of the results resembling a scenario that models one specific aspect of obesity or metabolic disease: overabundance of substrates to the mitochondria (23). In relation to DN and CKD, there is limited information available on carbon trafficking, substrate overabundance and on the effects of dysfunction of mitochondrial enzymes regulating such functions in the kidney. PTC relies heavily on mitochondrial fatty acid oxidation for their energy need (6), but it is not entirely clear how deviations in fatty acid oxidation affect the tubules. Our previous work in cell culture showed that mitochondrial entry of fatty acids is required for PTC apoptosis, rather than just cytoplasmic lipid accumulation (38). Here, we provide evidence that
when cellular/mitochondrial acyl-CoA balance and thus mitochondrial carbon trafficking is disturbed, mice develop kidney disease. Pathological features included tubular dilation, proteinaceous casts, fibrosis, proteinuria and interestingly, enlarged glomeruli and copious amounts of glomerular scarring. Importantly, these changes can be attributed to the tubular deletion of CrAT and likely not caused by other whole body metabolic alterations as mice display normal body weight, fasting glucose and respond normally on glucose tolerance test. Thus, our results indicate that a specific metabolic defect, driven by the deletion of a single gene of an important metabolic enzyme in PTC mitochondria is sufficient to cause tubular disease. PT-CrAT mice recapitulate many features of metabolic disease-related nephropathies, but without the confounding factors deriving from changes in whole body metabolism during diabetes. Therefore, it is a useful model to exclusively study the effects of mitochondrial derailments and substrate overload, without a number of additional factors that would derive from a diabetes model. Notably, PT-CrAT mice presented some range in the severity of pathology. This cannot fully be attributed to the genetic background as mice were created on C57BL/6 background and the original Ggt-Cre mice (mixed Balb/cJ/C57BL background) were crossed into C57BL/6 background for generations during the development and breeding of the cohorts. It is however consistent with previous findings from other mouse models of renal disease or human nephropathies where varying degrees of phenotypic changes are a characteristic feature (39-42).

Our results are in good agreement with those obtained by skeletal muscle-specific crat deletion. Many of the short-chain acylcarnitines were present in significantly lower concentrations in the cortex and urine with a concomitant increase of several of the medium- to long-chain acylcarnitines. The reduction in the levels of the short chain acylcarnitines was more
striking than initially expected. Such result suggests that CrAT in the PTC is absolutely essential to maintain the levels of these metabolites. Reduction in the levels of some of the odd chain metabolites (C3 and C5 in particular) reveals a more global effect on substrate metabolism, as these acylcarnitines are derived primarily from branched chain amino acid metabolism. Plasma levels of most of the acylcarnitines examined were not altered significantly, indicating that other tissues (such as skeletal muscle) are more likely significant contributors of circulating acylcarnitine levels. In healthy individuals, carnitine esters are excreted into the urine. Impairments in acylcarnitine excretion occur with deteriorating renal function (43). Thus, our findings of altered acylcarnitine profiles in the PT-CrAT mice may also suggest slowly declining renal function when PTC mitochondrial carbon trafficking is disturbed. There is also resemblance to a type 2 diabetic muscle. In patients with type 2 diabetes, CrAT mRNA abundance is ~ 80% lower in skeletal muscle compared to healthy individuals (23). In diabetic rats, accumulation of long-chain acylcarnitines was accompanied by lower free carnitine levels (44). Carnitine loss to the urine in our model thus suggests that there may be lower free carnitine levels in the kidney, or that there is carnitine loss due to an increase in injured PTC in the PT-CrAT mice. Accumulation of long-chain intermediates was coupled with depletion of several of the organic (TCA) intermediates. This observation is consistent with earlier ones in different organs where heightened β-oxidation appeared to reduce the levels of TCA intermediates (22). Such results may reflect compromised mitochondrial status. Markedly reduced pyruvate and lactate levels also suggest impairments in carbohydrate metabolism.

Susztak et al. studied derailments in fatty acid oxidation focusing on the mitochondrial FA import side. Their studies demonstrate lower expression of carnitine palmitoyl-transferase (Cpt1a) in kidneys from DN patients or in mice with tubulointerstitial fibrosis (10). Cpt1
facilitates the import of long-chain fatty acyl-CoAs into the mitochondria for \( \beta \) oxidation (45; 46). Defects in fatty acid oxidation due to downregulation or inactivity of Cpt1 are detrimental to the tubules. In light of the data of the Susztak group and ours, it seems that not only defective mitochondrial fatty acid oxidation but also a failure to remove excess acetyl-CoA and acylcarnitine products promotes kidney disease. As acetyl-CoA is a central metabolic intermediate, acetyl-CoA/CoA imbalance in the PTC appears to affect all three major nutrient metabolism pathways. As the glycolytic ability of the PTC is not significant and thus “substrate switching” – unlike in skeletal muscle – is not prominent in the tubules, we propose that PTC can handle an abundance of lipid-based substrates as long as the mitochondrial influx or efflux capacity of substrates/products are not compromised. Both Cpt1 and CrAT deficiencies seem to accompany the development of type 2 diabetes, and therefore could be significant contributors to tubular damage in CKD/DN.

Regarding ROS production, a prevailing theory is that acetyl-CoA is the main fuel for the TCA cycle, but overabundance of acetyl-CoA will produce reducing equivalents (NADH and FADH\(_2\)) in excess of that which can be handled by the ETC (22; 25). Such excess in turn creates an environment conducive to oxidative stress through the “backflow” of electrons from Complex III towards Complex I (25,47). Such redox pressure may exist regardless of whether TCA activity is increased (early DKD) or decreased (late DKD) (19) since heightened \( \beta \)-oxidation will provide excess NADH and FADH\(_2\). The “backflow” can change the reduced/oxidized state of the ETC complexes, leading to slower or diminished ATP production as a potential mechanism contributing to PTC energy deficit. Our data confirms that CrAT ablation increases the steady-state levels of lipid peroxide radicals as well as their end-products, 4-HNE in kidney cortex. Our results also suggest that in a lipid-rich environment such as the mitochondria, with ROS
production sites in close proximity to membrane structures, the odds are increased for lipid
radical production and lipid peroxidation. However, this may not be the only mechanism
contributing to cell damage or may only be limited to the mitochondria. Excess acetyl-CoA can
also drive acetylation of proteins, particularly the proteins of the ETC. In this regard, lysine
acetylation of mitochondrial proteins has been gaining attention as a prominent posttranslational
modification causing cellular stress, where the buffering capacity of CrAT was highlighted (47).
Modification of ETC proteins could then cause a slowdown in ATP production. While there
were no detectable differences in the levels of Lys acetylated proteins between control and CrAT
ablated mice in kidney cortex homogenates (Fig. 6C), acetylation as a posttranslational
modification is still worthy of further investigation. Detailed analysis in isolated mitochondria or
challenging permeabilized mitochondria with AcCoA+carnitine addition may reveal differences
in how mitochondrial overload can trigger such modifications.

Our data also reveal mitochondrial energy deficit and respiratory impairments as a
potential mechanism leading to tubular injury, as these alterations are present before the onset of
significant pathology. Primary PTCs isolated from the PT-CrAT mice at around 9 months of age
grew well in hormonally defined DMEM. At the same cell density however, their mitochondrial
respiration was significantly lower than those isolated from littermate fl/fl mice (Fig. 6). Most
importantly, PTC from PT-CrAT mice displayed energy deficit and lower ATP-linked
respiration suggesting that indeed, tubular mitochondrial acetyl-CoA imbalance leads to
derailments in mitochondrial energy production. If PTC mitochondria are not able to meet the
energy need of tubular cells, this could lead to significant deficits in for example tubular
epithelial function such as Na⁺/K⁺-ATPase activity. It is noteworthy that we only tested these
primary cells respirating on pyruvate, therefore, we cannot conclude whether PTCs lacking
CrAT would respirate differently on other substrates such as for example succinate or glutamate/malate. As it has been proposed before that excess acetyl-CoA is able to block PDH activity (23), a possible interpretation of our result is that PTCs with CrAT ablation cannot efficiently catabolize pyruvate. Mitochondrial content and abundance of ETC complexes was not different between normal and CrAT ablated mice, however, activities of complex I and complex V were reduced. It is therefore likely that either functionality or oxidized/reduced state of such complexes and/or one or more of their subunits are altered. Further investigation into mitochondrial structure, pathways and function is warranted, which was ongoing in our laboratory at the time of this manuscript was finalized. This includes electron microscopy analysis of mitochondrial structure, functional analysis of PT-CrAT tubular cells and a detailed next generation sequencing analysis of PT-CrAT cortices along a full timeline from 3 to 24 months to understand causality.

Our findings about secondary glomerulosclerosis are puzzling. Since CrAT has only been deleted in the proximal tubules, we suspect this is an indirect effect resulting from initial tubular damage. Also noteworthy that regardless of the variability of the phenotype (mild to more severe in the PTC), we consistently found secondary sclerosis in all of our cohorts. Similar findings have been reported before. Bonventre et al. found that repeated, selective tubular epithelial injury leads to secondary glomerulosclerosis (48). Authors surmised that such effect could be attributed to a paracrine signaling mechanism derived from injured/regenerating epithelium, or to a reduction of glomerular blood flow as a result of decreased loss of capillaries in tubulointerstitial fibrosis. It is also possible that a progressive tubulointerstitial reaction may directly encroach upon the glomerular tuft, causing the narrowing and disconnection of the glomerulotubular
junction (49-51). These results warrant further studies to determine the exact cause of
glomerulosclerosis observed in the PT-CrAT model.

CrAT deletion also further sensitized mice to high fat diet-induced renal injury. We
surmise that this is because of excess substrate due to the chronic lipid exposure further
compromises mitochondrial acetylcaritnine and short chain acylcarnitine efflux capacity. These
metabolites, together with a further surplus of reducing equivalents may block several metabolic
enzymes (through allosteric inhibition) and mitochondrial ETC proteins. The result is an
exacerbated overload impacting the kidney.

Taken together, PTC-specific ablation of CrAT causes tubular injury, glomerulosclerosis
and kidney disease in mice. We believe our results are in agreement with those supporting the
view that proximal tubular damage is also a primary, rather than just a secondary event in early
DN. The results are consistent with mitochondrial overload, showing chronic effects in vivo:
impaired nutrient utilization, which is not limited to fatty acid oxidation. These results provide
the basis for future studies to decipher the acute effects and a potential protection from overload.
This can be achieved for example through overexpressing CrAT in cultured tubular cells, then
challenging these cells to overload. In vivo however, such approach may not provide additional
protection in case of an abundant metabolic enzyme like CrAT. Our in vivo results point to a
much broader approach to tackle metabolic disease-associated tubular damage through
improving mitochondrial energy balance. We propose that disturbances in all three major
metabolic pathways (fatty acid, carbohydrate, amino acid) can prime the development of
DN/CKD, primarily through alterations in mitochondrial function, where lesions may occur first
in the tubules. Our findings may also have clinical implications: provided that mitochondrial
overload-induced mechanisms are the forerunners of PTC apoptosis, targeting mitochondrial
function and/or energy metabolism can emerge as a new area considered for future interventions
to effectively prevent, rather than treat tubular injury and CKD.

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DISCLOSURES

None.
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Figure legends

Figure 1. Generation of PTC-specific CrAT knockout mouse and baseline whole body parameters. (A) Genetic strategy to generate mice with PTC-specific ablation of CrAT (“PT-CrAT” mouse). Deletion was confirmed by (B) PCR in tail DNA, (C) CrAT knockout kidney cortices (but not heart, skeletal muscle, or eWAT) showed a ~2.6-fold reduced expression of CrAT mRNA (n=6, *p<0.006, Gapdh reference gene), (D) Western blot analysis of CrAT protein levels in kidney cortices showed successful deletion in the PT-CrAT mice. Kidney and skeletal muscle tissues from mice overexpressing CrAT under the β actin-promoter (“BAP-CrAT”) were used as positive control. (E) Enzyme activity was measured in whole kidney homogenates (residual CrAT activity derives from cells other than PTC). (F) PTC-specificity was verified by staining for CRE activity with an anti-Cre antibody and (G) by breeding PT-CrAT female mice with a tdTomato male. Red fluorescence around green brush borders stained positive by *Lotus tetragonolobus* lectin confirms PTC-specific Cre activity. Scale bar = 50 µm. (H) PT-CrAT mice body weights, (I) kidney weights, (J) fasting blood glucose levels and (K) glucose tolerance test (males, n=6-8).

Figure 2. CrAT deletion in PTC causes kidney disease. (A) Representative microphotographs of PAS and TriChrome staining in fixed, paraffin embedded fl/fl kidneys showing normal histology, (B) PT-CrAT kidneys show numerous protein casts in tubular lumen, tubular dilation (*), lipid droplets in tubular cells, TriChrome positive material/fibrosis (yellow arrows) and secondary glomerular disease ($) (≥18 months). (C) Tubular injury area was evaluated semiquantitatively and scores expressed as: minimal 1: <5 % of cortex, mild 2.5-24%, moderate 3.25-49%, severe 4:>50% of cortex. **p<0.005, n=6. (D) Serum creatinine levels in fl/fl and PT-
CrAT mice (n=4-10, *p<0.05). (E) Urinary albumin/creatinine ratios (males, n=8-12, **p<0.005) and urine protein analysis by electrophoresis/Coomassie blue staining (arrow points at albumin, ~ 67 kDa) in PT-CrAT mice compared to fl/fl controls, with equal amounts of protein (20 µg) loaded. (F) Collagen IV (cyan: ColIV, blue: DAPI) and (G) vimentin (red: Vim, blue: DAPI) positive staining in PT-CrAT tubules and glomeruli. (Photographs are from mice with the highest tubular injury scores). (H) Active caspase-3 staining (red: cleaved casp-3, green: *Lotus tetragonolobus*) in fl/fl and PT-CrAT kidneys. Scale bars = 50 µm. (I) Percentage of apoptotic PTC was evaluated by counting cleaved caspase-3 positive, *Lotus* counterstained cells. *p<0.05, n=4. (J) Gene expression analysis in fl/fl and PT-CrAT kidney cortices. *p<0.05, #p<0.01, &p<0.0005 vs fl/fl, n=6, male mice.

**Figure 3.** Altered acylcarnitine and organic acid profiles in the PT-CrAT model. (A) Kidney cortex, (B) plasma and (C) urine samples from PT-CrAT mice and fl/fl controls at 18-24 months of age were analyzed by tandem mass spectrometry for short- (SCAC), medium- (MCAC), and long-chain (LCAC) acylcarnitines, as well as (D) total and (E) free carnitine levels. (F) shows free/total carnitine ratios indicating urinary wasting of free carnitine. (G) Kidney cortices were analyzed for organic acid intermediates. *p< 0.05, ** p<0.005 vs. fl/fl. MS/MS analysis was normalized to show mice with the strongest kidney injury histology scores.

**Figure 4.** PT-CrAT mice have increased lipid-derived free radical levels. (A) Representative spectra of POBN-lipid radical adducts detected by ESR from Folch extracts of kidney cortex of PT-CrAT mice and fl/fl littermates (n=5). Increases in the amplitude of the six line spectrum peaks (arrows) are proportional to an increase in carbon-centered lipid-derived free radical.
levels. Spectra show a ~3-fold increase of lipid radicals in the PT-CrAT mice vs fl/fl control. (B) 4-hydroxynonenal-protein adducts were detected by Western blot analysis and (C) band intensities (normalized to β-actin as loading control) were measured using Image J (n=4, * p<0.05 vs. fl/fl).

**Figure 5.** A high fat diet accelerates kidney disease in the PT-CrAT mice. Representative pictures of PAS and TriChroem stained kidneys of (A) littermate fl/fl mice showing normal histology, (B) fl/fl mice kept on HFD and (C) PT-CrAT mice on HFD showing tubular damage (*), fibrosis and TriChroem positive material (yellow arrows), as well as glomerular sclerosis ($) and glomerulomegalia (12-15 months). (D) Glomerular tuft size was measured in each group (n=30-50 glomeruli per group, at 12 months of age). (E) Gene expression analysis in kidney cortices, (F) body weights, (G) plasma triglyceride and (H) free fatty acid levels in each experimental group. (n=4-6). # p<0.05 vs. fl/fl on chow, * p<0.05 vs. PT-CrAT on chow diet.

**Figure 6.** Primary PTCs from PT-CrAT mice have early alterations in mitochondrial respiratory function. PTCs isolated from fl/fl and PT-CrAT mouse kidneys (9 months) were grown on SeaHorse XF24 plates (75,000 cells/well). (A) Mitochondrial OCR was measured at baseline, and after the addition of 2 µM oligomycin, 2 µM FCCP or 1.5 mg/ml antimycin A. (B) Mitochondrial respiratory parameters were calculated and compared from the graph shown on (A). n=10, * p<0.05 vs. fl/fl. (C) Total acetylated lysine levels, (D) abundance of mitochondrial ETC complexes and (E) activity of mitochondrial complex I and V in kidney cortices of fl/fl and PT-CrAT mice (n=4, * p<0.05 vs. fl/fl).
Figure 7. A proposed scenario of mitochondrial overload in PTC. Mitochondrial overload modeled by CrAT deletion causes acetyl-CoA/free CoA imbalance and leads to the accumulation of incompletely oxidized products. Such imbalance impacts all three major metabolic pathways in PTC: fatty acid, amino acid and carbohydrate metabolism. As shown by our mass spectrometry results, overload also affects TCA cycle metabolite levels. Altogether these metabolic disturbances can contribute to PTC energy deficit. Furthermore, overload can cause mitochondrial redox imbalance through impacting the electron transport chain. Increased ROS and lipid peroxide production then also potentially contributes to PTC apoptosis.
Removal of exons 9-11. Mate with Ggt CRE-mice.

Removal of neo. (Mate with EIIa-CRE mice)

"PT-CrAT"

CrAT mRNA expression

Fasting glucose (mg/dL)

Blood glucose (mg/dL)

Kidney weight (g)

Relative CrAT activity

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Figure 2

A. PAS staining
B. TriColor staining
C. Tubular score
D. Serum creatinine
E. Western blot
F. Vimentin/DAPI
G. Relative mRNA
H. Caspase-3
I. Cleaved caspase-3 + tubular cells (%)
J. Relative mRNA

Diabetes
fl/fl
PT-CrAT

Serum creatinine (mg/dL)

Relative mRNA
Vim Col4A1 Bax Casp3

Cleaved caspase-3 + tubular cells (%)
C  Urine

**SCAC**

|        | fl/fl | PT-CrAT |
|--------|-------|---------|
| C2     |       |         |
| C3     |       |         |
| C4     |       |         |
| C4-OH  |       |         |
| C5     |       |         |
| C6     |       |         |

**MCAC**

|        | fl/fl | PT-CrAT |
|--------|-------|---------|
| C8     |       |         |
| C7DC   |       |         |
| C8DC   |       |         |
| C10    |       |         |
| C12    |       |         |

**LCAC**

|        | fl/fl | PT-CrAT |
|--------|-------|---------|
| C14    |       |         |
| C16    |       |         |
| C18:2  |       |         |
| C18:1  |       |         |
| C18    |       |         |

D  Free carnitine

E  Total carnitine

F  Free/total carnitine

**Figure 3**

For Peer Review Only
Kidney cortex

- Citrate, a-ketoglutarate, fumarate
- Succinate, pyruvate, malate, lactate

- fl/fl
- PT-CrAT

pmol/mg tissue

* denotes statistical significance.
Figure 4

A B C

PT-CrAT fl/fl

4-HNE-adducts

actin PT-CrAT fl/fl

Relative band intensity (a.u.)

PT-CrAT fl/fl
Figure 5

A. fl/fl
B. fl/fl + HFD
C. PT-CrAT + HFD

**PAS**

**TriChrome**

**Body weight (g)**

**TG (mg/dL)**

**FFA (μM)**

**Relative mRNA**

**Diabetes**
Figure 6

(A) OCR (pmol/min) vs. Time (min)

(B) Basal, ATP-linked Maximal Reserve H+ leak

(C) Acetyl-Lys

(D) Relative band intensity (a.u.)

(E) V_{Max}(NADH) (control %)
Figure 7: Proximal tubular cell energy deficit

- Fatty acids
  - β-oxidation
- Amino acids
- Acetyl-CoA/CoA imbalance
- Carbohydrates
  - PDH
- TCA cycle disturbance
- CrAT

Accumulation of intermediate (LCAcCarn) products

Mitochondrial redox imbalance

- Reaction with lipids in the membrane
- ROS mediated PTC apoptosis

\[ \text{Fe}^{2+} \rightarrow \text{O}_2^\cdot \rightarrow \cdot\text{OH} \rightarrow \text{LOC}^\cdot \]