Proximal Tubular Cell-Specific Heterozygous Ablation of Carnitine Acetyltransferase Causes Cellular and Respiratory Remodeling as a Component of Tubular Disease

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Introduction

Proximal tubular epithelial cells are highly energy-demanding, and they derive most of their energy from mitochondrial fatty acid oxidation. Since mitochondrial dysfunction has been suggested as a precursor to tubular damage, this lab has modeled mitochondrial overload by studying mice lacking the enzyme carnitine acetyltransferase (CrAT) in the proximal tubules. CrAT is a mitochondrial matrix enzyme that removes excess substrates from the mitochondrial matrix by linking primarily short-chain acetyl-CoA products to carnitine, rendering them membrane-permeable. Ablation of CrAT mimics the mitochondrial substrate-overload aspect of metabolic disease by causing acetyl-CoA accumulation in the mitochondrial matrix, resulting in an acetyl-CoA/CoA imbalance. This specific ablation of CrAT offers a useful model for diabetic renal disease-induced lipotoxicity without the confounding factors of metabolic disease.

Abstract

This lab has previously shown that proximal tubule cell-specific homozygous knockout of CrAT (PT-CrAT^{HOM}) is associated with energy deficit and impaired respiration before the onset of pathology, suggesting that mitochondrial dysfunction may be causative to chronic kidney disease (1). Respiratory and ultrastructural effects of heterozygous ablation of CrAT (PT-CrAT ^{HET}) seems to have as profound effects as homozygous ablation, as evidenced by recent functional and histological studies. This highlights the importance of even mild inhibition of CrAT observed in states of lipid stress in the progression of renal disease (2). Our analysis of transmission electron microscopy (TEM) images of PT-CrAT^{HET} kidneys in mice aged 12-16 months reveals more numerous lipid droplets, extensive deterioration of mitochondrial architecture, increased levels of mitophagy, and the presence of multilamellar bodies compared to control mice. Ongoing respiratory studies involve providing isolated PT-CrAT^{HET} segments with pyruvate to investigate how the segments respirate differently with only 1 functional CrAT allele using a XF24 Analyzer (Agilent Seahorse) (3). Since it has been proposed that excess acetyl-coA can inhibit pyruvate dehydrogenase, it was hypothesized that these segments would catabolize pyruvate less efficiently than control segments.

Methods

- **Animals.** Mice with targeted deletion of CrAT in PTC were generated using Cre-loxP recombination strategy. Homozygous CrATloxP female mice created by Dr. Randall Mynatt at the Pennington Biomedical's Transgenic Core were bred to male γ-glutamyl-transferase Cre mice (*Tg-(Ggt1-cre)M3Egn/J*, 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 create the haploid insufficiency, homozygous PT-CrAT mice were crossed to wild type mice to create the PT-CrAT HET mice.
- **Histology.** Paraffin embedded kidneys were cut into 5 µm cross-sections at the Pennington Biomedical's Cell Biology and Imaging Core Facility. Sections were mounted on charged SuperFrost slides (Fisher Sci.), and deparaffinized before staining. Sections were stained with a) Periodic acid-Shiff (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.
- **Primary PTC segments and mitochondrial respiration analysis.** Kidneys from PT-CrAT HET and fl/fl mice were harvested and placed into ice cold Krebs-Henseleit buffer for primary proximal tubular epithelial cell isolation. PTCs were isolated using a collagenase digestion and sequential sieving method originally described by Vinay et al. Cells were grown in hormonally defined DMEM media. Protein content was measured from the pellet and equalized then PTC segments were plated onto 24-well XF24e SeaHorse culture plates. Mitochondrial oxygen consumption was measured in cells respirating on 5 mM pyruvate using a SeaHorse XF24 Extracellular Flux Analyzer.
- **Serum creatinine**. Creatinine levels in serum were measured using a mouse Creatinine kit (Cayman Chemicals).
- **TEM**. Kidneys were fixed in Karnovsky's fixative from all experimental groups. The cortex was separated from medulla, and cut into 1 mm^3 sized cubes. Samples were analyzed using a Jeol Transmission Electron Microscope. The outcomes indicating mitochondrial alterations are: mitochondrial swelling, fragmentation, round mitochondria (instead of elongated ones) with disorganized cristae, lipid droplets, and droplets with double membrane structure (sign of autophagic activity). We compared at least 20 viewing areas per sample, to determine the numbers and ratio of normal vs. abnormal mitochondria and number of lipid droplets in each group. Total counts were compared using Student's *t*-test, at p<0.05.

Figure 1. Mitochondrial substrate overload is a result of nutrient excess in metabolic disease and Type II Diabetes Mellitus

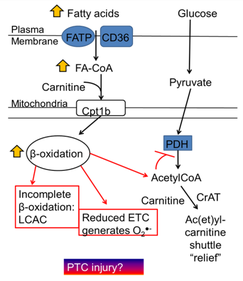
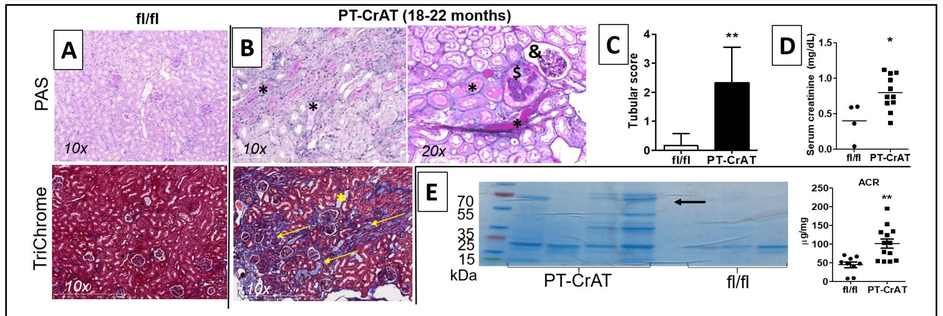
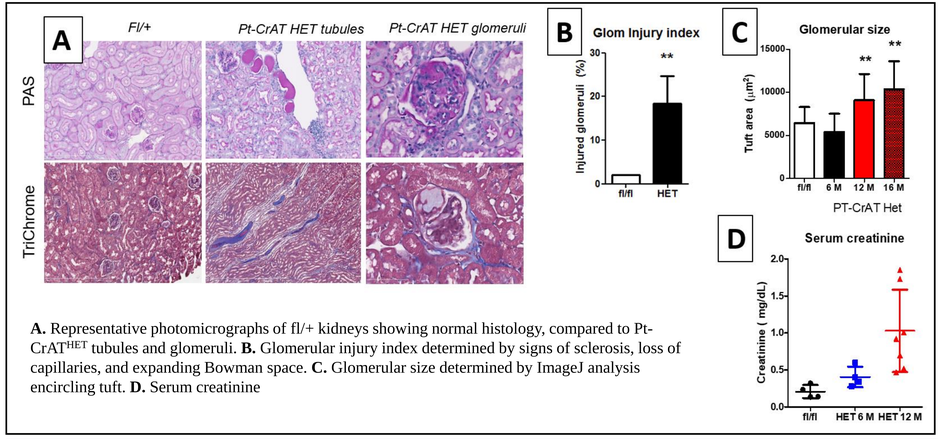


Figure 2. PT-CrAT^{HOM} mice develop kidney disease



A: Representative photomicrographs of 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 are expressed as minimal 1: ,5% of cortex, mild 2: 5–24%, moderate 3: 25–49%, and severe 4: .50% of cortex (n = 6). **P, 0.005. **D**: Serum creatinine levels in fl/fl and PT-CrAT mice. **E**: Urine protein analysis by electrophoresis/Coomassie blue staining (arrow points at albumin, ;67 kDa), and urinary albumin-to-creatinine ratios (ACR) (males, n = 8–12). **P,0.005. Staining positive for collagen IV (cyan: collagen IV, blue: DAPI)

Figure 3. PT-CrAT^{HET} mice exhibit similarly severe kidney disease as PT-CrAT^{HOM} mice



A. Representative photomicrographs of fl/+ kidneys showing normal histology, compared to Pt-CrAT^{HET} tubules and glomeruli. **B.** Glomerular injury index determined by signs of sclerosis, loss of capillaries, and expanding Bowman space. **C.** Glomerular size determined by ImageJ analysis encircling tuft. **D.** Serum creatinine

References & Acknowledgements

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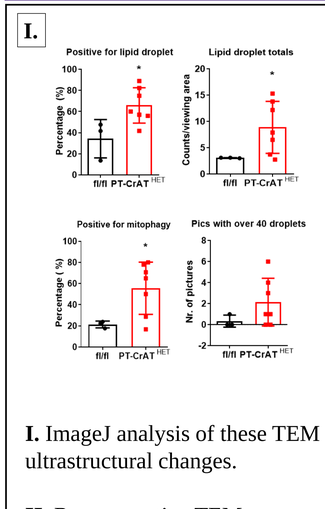
2. Seiler SE, Martin OJ, Noland RC, Slentz DH, DeBalsi KL, Ilkayeva OR, An J, Newgard CB, Koves TR, Muoio DM. Obesity and lipid stress inhibit carnitine acetyltransferase activity. Journal of Lipid Research 2014;55:635-644.
3. McCrimmon A, Domondon M, Sultanova RF, Ilatovskaya DV, Stadler K. Comprehensive assessment of mitochondrial respiratory function in freshly isolated nephron segments. Am J Physiol Renal Physiol 2020;318:F1237-F1245.

- We thank Dr. Daria Ilatovskaya for providing us with the TEM images and guidance for TEM ImageJ analysis.

Figure 4. PT-CrAT^{HET} cells exhibit more numerous lipid droplets and more mitophagy as an early sign of tubular disease



I. ImageJ analysis of these TEM ultrastructural changes.

II. Representative TEM photographs of mitochondrial ultrastructural changes in fl/fl and PT-CrAT^{HET} kidneys.

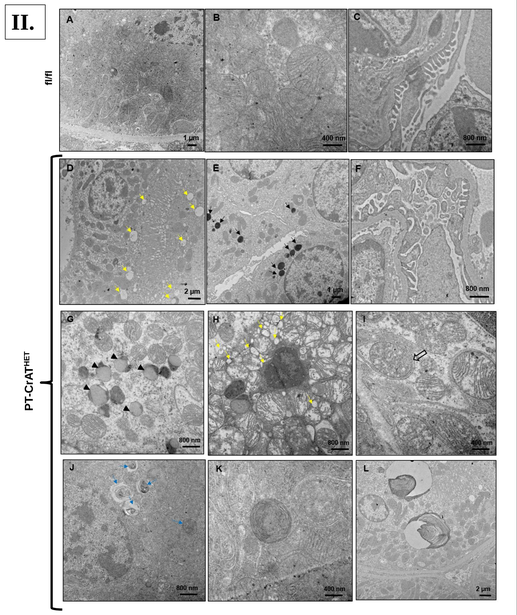
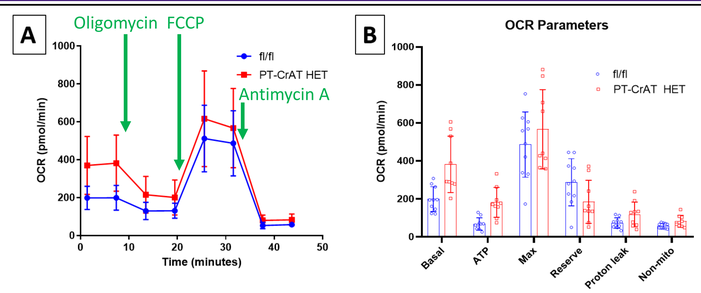


Figure 5. PT-CrAT^{HET} cells exhibit greater basal OCR and decreased reserve capacity



A. Oxygen Consumption Rate (OCR) data for freshly isolated proximal tubules from a pair of male mice aged 17-19 months respirating on pyruvate

B. GraphPad analysis of OCR data.

Conclusions and Future Directions

- Histological analysis of PT-CrAT^{HET} cells indicates that intact mitochondrial substrate efflux is essential in the prevention of chronic kidney disease because having only one functional CrAT allele elicits robust ultrastructural evidence for cellular damage.
- Contrary to the original hypothesis, our data suggest that PT-CrAT^{HET} mitochondria exhibit greater basal oxygen consumption rate when respiring on pyruvate. Possible explanations include:
  - PT-CrAT^{HET} may exhibit greater basal proton leak, indicating a possible greater amount of uncoupled respiration and cellular reactive oxygen species compared to control mitochondria.
  - PT-CrAT^{HET} mitochondria also exhibit less reserve respiratory capacity, indicating they may not be able to handle oxidative stress as well as control mitochondria. These ongoing respiratory studies will offer insight into mitochondrial function in the progression of tubular injury leading to pathology and will inform future time-course and male-versus-female investigations.
- The current studies, taken together with this lab's recent 2019 publication (1), suggest that normalizing tubular cell mitochondrial function may serve as an important preventative strategy against chronic kidney disease.