Inhibition of amino acids influx into proximal tubular cells improves lysosome function in diabetes.

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Key Points

- Collectrin (CLTRN) serves as a chaperone for the trafficking of neutral amino acid transporters in the apical membranes of proximal tubular cells (PTCs).
- *Cltrn* knockout reduced amino acids influx into PTCs, inactivated mTOR, activated TFEB, improved lysosome function, and ameliorated vacuolar formation of PTCs in diabetic mice treated with streptozotocin and high fat diet.
- The inhibition of neutral amino acid transporter, such as B^0AT1 (SLC6A19), and TFEB activator is a new therapeutic strategy against diabetic kidney disease.

Abstract

Background/aims: Inhibition of glucose influx into proximal tubular cells (PTCs) by sodium-glucose co-transporter 2 (SGLT2) inhibitors revealed prominent therapeutic impacts on diabetic kidney disease (DKD). Collectrin (CLTRN) serves as a chaperone for the trafficking of neutral amino acid transporters in the apical membranes of proximal tubular cells. We investigated the beneficial effects of reduced influx of amino acids into proximal tubular cells in diabetes and obesity model of Cltrn-/y mice.

Methods: Cltrn+/y and Cltrn-/y mice at 5 weeks of age were assigned to standard diet- (STD) and streptozotocin and high fat diet-treated (STZ-HFD) groups.

Results: At 22-23 weeks of age, body weight and HbA1c levels significantly increased in STZ-HFD-Cltrn+/y compared to STD-Cltrn+/y; however, they were not altered in STZ-HFD-Cltrn-/y compared to STZ-HFD-Cltrn+/y. At 20 weeks of age, urinary albumin creatinine ratio (UACR) was significantly reduced in STZ-HFD-Cltrn-/y compared to STZ-HFD-Cltrn+/y. Under the treatments with STZ and HFD, the *Cltrn* gene deficiency caused significant increase in urinary concentration of amino acids such as Gln, His, Gly, Thr, Tyr, Val, Trp, Phe, Ile, Leu and Pro. In proximal tubular cells in STZ-HFD-Cltrn+/y, the enlarged lysosomes with diameter of 10 μm or more were associated with reduced autolysosomes, and the formation of giant lysosomes was prominently suppressed in STZ-HFD-Cltrn-/y. Phospho-mTOR and inactive form of phospho-TFEB were reduced in STZ-HFD-Cltrn-/y compared to STZ-HFD-Cltrn+/y.

Conclusions: The reduction of amino acids influx into proximal tubular cells inactivated mTOR, activated TFEB, improved lysosome function, and ameliorated vacuolar formation of PTCs in STZ-HFD-Cltrn-/y mice.

Introduction

Collectrin is a 222-amino acid (AA) transmembrane glycoprotein and angiotensin converting enzyme 2 (ACE2) homologue. Although ACE2 shares collectrin domain at C-terminal end, collectrin lacks the active dipeptidyl carboxypeptidase catalytic domains located at N-terminal of ACE and ACE2[1]. Collectin is highly expressed in an apical brush border of PTCs[2, 3], collecting duct cells[1], and pancreatic β cells[4]. Collectrin is one of the downstream target genes of hepatocyte nuclear factor-1α (HNF-1α) and the overexpression of a dominant-negative mutation form (HNF1α-P291fsinsC) derived from the patients with type 3 form of maturity-onset diabetes of the young (MODY3) in INS-1 pancreatic β cells resulted in the reduced expression of collectrin[5]. In the patients with MODY3, hyperglycemia increases over time, resulting in the need for treatment with oral hypoglycemic drugs or insulin. Collectrin binds to soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex consisting of synaptosomal-associated protein, 25kDa (SNAP25), vesicle associated membrane protein 2 (VAMP2), and syntaxin 1 via direct interaction with snapin and facilitates the glucose-stimulated insulin exocytosis. In rat insulin promoter (RIP)-collectrin transgenic mice, the reduced blood glucose levels associated with enhanced insulin secretion of in the glucose tolerance tests were reported[4]. The patients with MODY3 also develop Fanconi's syndrome and manifest glucosuria and generalized aminoaciduria. The collectrin knockout mice develop severe generalized aminoaciduria accompanied with reduced expression of AA transporters, such as SLC6A19 (B^0AT1; XT2s1) for neutral/aromatic AAs, SLC6A18 (B^0AT3; XT2; Xtrp2) for Gly/Gln, SLC6A20 (IMINO; SIT1; XT3; Xtrp3) for imino acids (Pro) at the apical membrane of PTCs, suggesting collectrin serves as a chaperone for the trafficking of amino acid transporters in the apical membranes of PTCs[2, 3].

Since collectrin was up-regulated in 5/6 nephrectomized mice and Wistar-Kyoto rats fed with high-salt diet, it suggested possible role of collectrin in salt-sensitive hypertension. In the initial research, on a mixed genetic background of 129/SvEv × C57BL/6J, a statistically significant difference in arterial pressure compared to wild-type (WT) mice was not detected[6]. The 129/SvEv strain is more susceptible to the development of hypertension than the C57BL/6J and collectrin deficiency resulted in severe hypertension associated with augmented salt sensitivity and impaired pressure natriuresis[7]. Collectrin is also expressed in endothelial cells, maintains plasma membrane levels of cationic amino acid transporter 1 (CAT1) and L-type amino acid transporter 1 (y(+)LAT1), facilitates L-arginine uptake and NO production by endothelial nitric oxide synthase. Recently, Le TH *et al*. reported loss of collectrin in the proximal tubule is sufficient to induce hypertension and proximal tubule specific knockout mice exhibited hypertension associated with increased sodium-hydrogen exchanger 3 (NHE3) expression and compensatory enhanced endothelium-mediated dilatation without exacerbation of salt sensitivity[8].

Here, we investigated Cltrn-/y mice backcrossed to C57BL/6JJcl, the strain resistant to salt-sensitive hypertension and sensitive to development of diabetes and obesity, by inducing strepotozotocin (STZ) and high fat diet (HFD) model of diabetes. We aimed to focus on the beneficial effects of reduced influx of amino acids into proximal tubular cells in Cltrn-/y mice by using this animal model characterized by increased influx of nutrients into proximal tubules.

Methods

Animal Models

We obtained Cltrn-/y male and Cltrn+/- female mice (B6;129S5-*Cltrn*^{tm1Lex}Mmucd, MGI:5007377) from MMRRC (Mutant Mouse Regional Resource Centers) at UC Davis

(Davis, CA, USA). *Cltrn* gene consists of 7 exons and exons 1 and 2 were targeted (NM_020626.1) by homologous recombination. Lexicon cell line derived from 129S5/SvEvBrd was used as donor strain and the mice were mated with C57BL/6JJcl to produce F5 mice and used for following experiments. The PCR genotyping was performed using wild-type-specific primers (3: 5'-TGCAGGGGCACTAGTACAGATC-'3 and 12: 5'-CTCATTCTATCAGTGACTCCC-'3) and mutation-specific primers (Neo3a: 5'-GCAGCGCATCGCCTTCTATC-3' and 2: 5'-CTCATTCTATCAGTGACTCCC-'3), and size of predicted PCR products were 365 bp and 300 bp, respectively.

C57BL/6 male mice fed high fat diet (HFD) and single dose of streptozotocin (STZ), a model of type 2 diabetes and diabetic kidney disease with increased Urinary albumin/creatinine ratio (ACR) were used[9]. Five-week-old mice were randomly assigned to standard diet (STD) group (D12450 [10 kcal% fat, 7 kcal% sucrose], Research Diets, New Brunswick, NJ) or STZ + HFD group (D12492 [60 kcal% fat, 7 kcal% sucrose], Research Diets), the latter received single injection of 100 mg/kg of STZ in 0.1 M citrate buffer, pH 4.5 (Sigma-Aldrich). The 13-week-old mice (n=4 in each experimental group) were fasted for 16 hours in glucose tolerance test (GTT) and for 3 hours in insulin tolerance test (ITT). They were then intraperitoneally injected with glucose solution (1 mg/g body weight) and human insulin (1 unit/kg in STZ+HFD and 0.75 unit/kg in STD groups) for GTT and ITT, respectively.

All animal experiments were approved by the Animal Care and Use Committee of the Department of Animal Resources, Advanced Science Research Center, Okayama University (OKU-2017218, 2017222, 2018208, 2018469, 2018470, 2018545, 2018666, 2021519, 2021579, and 2021699).

Western Blot Analysis

The kidney tissues from 35-week-old mice were homogenized in RIPA lysis and Extraction buffer plus Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher scientific). The samples were boiled in Laemmli Sample buffer, separated on 4-20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad), and transferred to a PVDF Blotting Membrane (cytiva). After blocking with 5% nonfat milk for 1 hour at room temperature (RT), the blots were incubated with primary antibodies diluted with Can Get Signal Solution 1 (TOYOBO); Phospho-mTOR (Ser2448) (D9C2) XP Rabbit Monoclonal Antibody (Cell Signaling Technology Cat# 5536, RRID: AB_10691552), mTOR (7C10) Rabbit Monoclonal Antibody (Cell Signaling Technology Cat# 2983, RRID: AB_2105622), Anti-phospho TFEB (Ser142) (Sigma-Aldrich Cat# ABE1971-I, RRID:AB_2928101), ATG13 antibody (Proteintech Cat# 18258-1-AP, RRID:AB_2130658), Anti-p62 (SQSTM1) Polyclonal Antibody (MBL International Cat# PM045, RRID:AB_1279301), Anti-LC3 Polyclonal Antibody (MBL International Cat# PM036, RRID:AB_2274121), Raptor (24C12) Rabbit mAb (Cell Signaling Technology Cat# 2280, RRID: AB_561245), p70 S6 Kinase (49D7) Rabbit mAb (Cell Signaling Technology Cat# 2708, RRID: AB_10694087), p70 S6 Kinase (49D7) Rabbit mAb (Cell Signaling Technology Cat# 2708, RRID: AB_10694087) overnight at 4°C. GAPDH (D16H11) XP Rabbit mAb (HRP Conjugate) (Cell Signaling Technology Cat# 8884, RRID: AB_11129865) was used as a loading control. After washing three times with Tris-buffered saline (TBS), the blots were incubated with ECL Donkey Anti-Rabbit IgG, HRP-Conjugated Antibodies (NA934V, GE healthcare Life science, 1:100,000) diluted with Can Get Signal Solution 2 (TOYOBO) at RT for 1 hour. The blots were developed with Pierce ECL Western Blotting Substrate (TE261327, Thermo Fisher Scientific). The chemiluminescence was analyzed using ImageQuant LAS-4000 mini (FUJIFILM).

Statistical Analysis

All values were represented as the mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism (version 8.0). Unpaired t tests, One-way and Two-way ANOVA with Tukey tests were used to determine the differences. $p<0.05$ was considered statistically significant.

Results

Glucose metabolism in STZ+HFD-Cltrn-/y mice

Under STD, there are no significant differences in body weight between STD-Cltrn+/y (34.6±2.5 g) and STD-Cltrn-/y (31.7±2.1 g) at 35 weeks of age. By STZ injection and HFD feeding, body weight significantly increased in STZ-HFD-Cltrn+/y (51.4±1.7 g) compared to STD-Cltrn+/y (34.6±2.5 g) at 35 weeks of age ($p=0.0041$). There were no significant differences in body weight between STZ-HFD-Cltrn+/y (51.4±1.7 g) and STZ-HFD-Cltrn-/y (44.1±8.1 g) at the final observation point of 35 weeks of age (**Figure 1A**). The weight of white adipose tissues (WATs) and liver significantly increased in STZ-HFD-Cltrn+/y compared to STD-Cltrn+/y at 35 weeks of age; however, there were no significant differences in weight of various organs between STZ-HFD-Cltrn+/y and STZ-HFD-Cltrn-/y (**Figure 1B**). There were no significant differences in systolic blood pressure in any timepoints (**Figure 1C**).

Ad libitum blood glucose levels were significantly reduced in STZ-HFD-Cltrn-/y (191±41 mg/dL) compared to STZ-HFD-Cltrn+/y (343±5 mg/dL) at 23 weeks of age ($p=0.0034$) (**Figure 2A**), and there was significant reduction in HbA1c levels in STZ-HFD-Cltrn-/y (5.0±0.45 %) compared to STZ-HFD-Cltrn+/y (6.2±0.40 %) ($p=0.0346$) at 23 weeks of age (**Figure 2B**). In GTT, there were no significant differences in blood glucose and serum insulin levels between STZ-HFD-Cltrn+/y and STZ-HFD-Cltrn-/y at 13 weeks of age (**Figure 2C and 2D**). In ITT, blood glucose levels were significantly reduced in STZ-HFD-Cltrn-/y (129±71 mg/dL) compared to STZ-HFD-Cltrn+/y (272±42

mg/dL) at 15 minutes after intraperitoneal injection of glucose (p=0.0474) (**Figure 2E**), and area under the curve (AUC) wad significantly reduced in STZ-HFD-Cltrn-/y (214±122 hr*mg/dL) compared to STZ-HFD-Cltrn+/y (433±127 hr*mg/dL) (p=0.0495) (**Figure 2E**). Overall, there were metabolic improvements in insulins sensitivity in STZ+HFD-Cltrn-/y compared to STZ+HFD-Cltrn+/y mice.

Oxygen consumption rate and locomotor activities in STZ+HFD-Cltrn-/y mice

Respiratory quotient was significantly reduced in the mice treated with STZ and HFD; however, there were no significant differences in STZ-HFD-Cltrn+/y (0.757±0.004 and 0.739±0.011) and STZ-HFD-Cltrn-/y (0.765±0.006 and 0.755±0.011) during light and dark periods, respectively (**Figure 3A and 3B**). Oxygen consumption rate significantly increased during dark periods in STZ-HFD-Cltrn-/y (0.080±0.009 mL/g/min) compared to STZ-HFD-Cltrn+/y (0.066±0.005 mL/g/min) (p=0.0458) (**Figure 3C and 3D**). By the treatment of STZ and HFD, locomotor activities were significantly reduced in STZ-HFD-Cltrn+/y (1.9±0.3 counts/min) compared to STD-Cltrn+/y (5.6±1.9 counts/min) (p<0.0001). Lowered locomotor activities were significantly and partially reversed by the deficiency of *Cltrn* in STZ-HFD-Cltrn-/y (3.7±0.9 counts/min) compared to STZ-HFD-Cltrn+/y (1.9±0.3 counts/min) (p=0.0209) (**Figure 3E and 3F**).

Urinary albumin excretion and urinary amino acids profile

Urinary albumin/creatinine ratio (ACR) significantly increased in STZ-HFD-Cltrn+/y (424±65 and 571±18 μg/mg) compared to STD-Cltrn+/y (193±48 and 193±40 μg/mg) at 14 and 20 weeks of age (p=0.0308 and p=0.0028), respectively (**Figure 4A**). ACR was significantly reduced in STZ-HFD-Cltrn-/y (276±78 μg/mg) compared to STZ-HFD-Cltrn+/y (571±18 μg/mg) at 20 weeks of age (p=0.0024) (**Figure 4A**). At 33 weeks of age, Kidney injury molecule-1 (Kim-1)/creatinine ratio (KCR) increased in STZ-HFD-

Cltrn+/y (18.3±5.19 pg/mg) compared to STD-Cltrn+/y (9.51±1.72 pg/mg) (p=0.0533). KCR was significantly reduced in STZ-HFD-Cltrn-/y (8.59±4.47 pg/mg) compared to STZ-HFD-Cltrn+/y (18.3±5.19 pg/mg) (p=0.0219) (**Figure 4B**). *Cltrn* deficiency resulted in amino acids uptake defects because of down regulation of apical amino acids transporters in PTCs in previous studies. We next investigated the urinary amino acids profile and influence of the treatments with STZ and HFD. By the multiple unpaired t tests between STD-Cltrn+/y and STD-Cltrn-/y, 17 amino acids significantly increased in STD-Cltrn-/y except Arg, Cys, and Lys (**Table 1**). In contrast, any amino acids concentrations were not altered by the treatment of STZ and HFD in the comparison between STD-Cltrn-/y and STZ+HFD-Cltrn-/y (**Table 1**). In the comparison between STZ+HFD-Cltrn+/y and STZ+HFD-Cltrn-/y, the *Cltrn* gene deficiency caused significant increase in urinary amino acids concentration of neutral amino acids such as Gln, His, Gly, Thr, Val, Trp, Phe, Ile, Leu and Pro by unpaired t tests (**Table 1**) and Gln, Gly, Thr, and Ala in Two-way ANOVA with Tukey tests (**Figure 4B**).

Morphological changes in mouse kidney tissues treated with STZ and HFD

Under feeding with STD, there were no discernible morphological changes in kidney tissues of STD-Cltrn+/y and STD-Cltrn-/y (**Figure 5A and 5B**) by light microscopic observations with PAS stain. However, prominent vacuolations were observed in tubular cells of STZ-HFD-Cltrn+/y (**Figure 5C,** red arrows) and they were mostly suppressed in STZ-HFD-Cltrn-/y (**Figure 5D**). The lysosome/total area % was significantly reduced in STZ-HFD-Cltrn-/y compared to STZ-HFD-Cltrn+/y (**Figure 4D**). The PTCs characterized by prominent apical microvilli formation and richness of mitochondria, and they were investigated by electron microscopy. In PTCs in STZ-HFD-Cltrn+/y, enlarged giant lysosomes with diameter of 10 μm or more were observed (**Figure 6A,** red arrows). The regular size of lysosomes with diameter of 1 μm

or less were also observed (**Figure 6A,** red arrowheads). Furthermore, various forms of autophagosomes were observed in higher magnification (**Figure 6A,** yellow arrowheads); however, autolysosomes were not observed in the section of single proximal tubular cell showing giant lysosomes. The proximal tubular cell sections without giant lysosomes were also observed in STZ-HFD-Cltrn+/y (**Figure 6C and D**), where many autolysosomes (**Figure 6D**, blue arrows) and lipid autolysosomes (**Figure 6D**, orange arrows) were observed. Electron microscopic observation suggested the lysosome stress, enlargement of lysosome, and the stagnation of the autolysosome formation in proximal tubules in STZ-HFD-Cltrn+/y. In STZ-HFD-Cltrn-/y, the formation of giant lysosomes was prominently suppressed (**Figure 6E**) as observed in light microscopy (**Figure 5D**). In higher magnification, the formation of both autophagosomes (**Figure 5F**, yellow arrowheads) and autolysosomes (**Figure 5F**, blue arrows) were observed in STZ-HFD-Cltrn-/y.

mTOR signaling pathways in STZ+HFD-Cltrn-/y mice

Since amino acids activate mammalian target of rapamycin complex 1 (mTORC1) and it also regulates autophagy by controlling lysosome biogenesis through phosphorylation of transcription factor EB (TFEB), we further investigated the kidney expression of phospho-mTOR, phospho-TFEB, autophagy related 13 (ATG13), p62, Microtubule-associated protein light chain 3 (LC3), Raptor, p70 S6 Kinase (S6K) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by Western blot analyses (**Figure 7A**). Both phospho-mTOR/mTOR and phospho-TFEB/GAPDH ratios were significantly reduced in STZ-HFD-Cltrn-/y compared to STZ-HFD-Cltrn+/y (**Figure 7B**). Decreased autophagic activity is reflected by the reduction of ATG13/GAPDH and LC3-II/LC-I accompanied with the accumulation of p62/GAPDH. There were no significant differences between STZ-HFD-Cltrn+/y and STZ-HFD-Cltrn-/y in ATG13/GAPDH, LC3-

II/LC-I, p62/GAPDH, Raptor/GAPDH, and S6K/GAPDH (**Figure 7B**). The data suggested that reduction of amino acids influx into proximal tubular cells activated mTOR and TFEB, which resulted in the improved lysosome function, formation of autolysosome and reduction of giant lysosomes in STZ-HFD-Cltrn-/y.

Discussion

The PTCs play a central role in the regulation of reabsorption, degradation, and production of the nutrients such as glucose, free fatty acids, and amino acids by sensing the nutrient signals from urinary space under the physiological conditions[10]. In the patients with diabetes and obesity, the excess of food intake results in the overload of glucose, free fatty acids and amino acids to PTCs and the enhanced activation of mTORC1 and AMP-activated protein kinase (AMPK) links the subsequent induction and progression of tubular injury, inflammation, and fibrosis[10]. Neutral amino acids are actively transported from lumen to cytoplasm by SLC6A19 (B0AT1; XT2s1), SLC6A18 (B0AT3; XT2; Xtrp2), and SLC6A20 (IMINO; SIT1; XT3; Xtrp3) clustered in the apical membranes of PTCs[2, 3]. Collectrin binds to snapin, facilitates the exocytosis of insulin in pancreatic β cells and could form the heterodimers with SLC6A19, SLC6A18, and SLC6A20, suggesting collectrin is responsible for the clustering of neutral amino acids transporters on the apical membrane of PTCs[11, 12]. In STZ-HFD-Cltrn-/y, urinary excretion of many of neutral amino acids significantly increased but acidic (Asp and Glu) and basic (Arg and Lys) amino acids were unaltered compared to STZ-HFD-Cltrn+/y. It enabled us to investigate the effects of reduced influx of most of the neutral amino acids into the PTCs under the state of diabetes and obesity.

mTOR acts as a serine/threonine kinase by interacting with multiple protein complexes such as mTORC1 and mTORC2. mTORC1 is consisted of DEPTOR, PRAS40, Raptor, mLST8/GbL, Tti/Tel2, and mTOR and its activation is initiated by

amino acid-mediated formation of active RagA/B-GTP and RagC/D-GDP heterodimers binding to Raptor followed by recruitment of mTORC1 to lysosome where Rheb activator locates, suggesting a dominant role of amino acids in the regulation of mTORC1[13]. The mechanism how the cells sense amino acids and transduce signals to mTORC1 is particularly investigated in Leu and Arg. Under the Leu starvation, Sestrin2 binds to GATOR2 and the complex inhibits the GTPase-activating protein activity of GATOR1, which results in the hydrolysis of RagA/B-GTP to GDP, dissociation of mTORC1 from lysosome, and its inactivation. Leu is recognized by Sestrin2, cytosolic sensor, dissociates from GATOR2, and induces the activation of mTORC1[14]. Cytosolic arginine sensors for mTORC1 subunit 1 (CASTOR1) regulates mTORC1 activity through a mechanism of action like Sestrin2. CASTOR1 functions as a homodimer or heterodimer with CASTOR2, which is required for arginine binding, GATOR2 sequestration and mTORC1 inactivation[15]. Gln is an important amino acid for activation of mTORC1 depending on α-ketoglutarate production in a Rag GTPases-dependent manner[16]. Gln also activates mTORC1 via v-ATPase and Arf1 in a Rag GTPases-independent manner[17]. In our animal model with diabetes and obesity, significant increase in urinary excretion of Gln and Leu in STZ-HFD-Cltrn-/y suggesting reduced influx Gln and Leu into PTCs contribute the ameliorated activation of mTOR, *i.e.* phosphorylation of mTOR, compared with STZ-HFD-Cltrn+/y. To support this notion, synergistic promotive effect of Gln on Leu-mediated mTORC1 activation has been reported[18].

STZ-HFD-Cltrn+/y demonstrated the increased phosphorylation of mTOR and we observed reversed activation of mTOR in STZ-HFD-Cltrn-/y associated with increased amino acids excretion into urine. We further investigated the status of autophagy downstream of mTORC1 in the kidney tissues. In STZ-HFD-Cltrn+/y, the PTCs demonstrates enlargement of lysosome associated with impairment of autolysosomes

formation. In Western blot analyses, ATG13 (autophagy initiation), p62 (autophagy substrate and marker for autophagy activity), and LC3 (autophagosome marker) demonstrated the impairment of autophagy process in STZ-HFD-Cltrn+/y; however, they were not reduced in STZ-HFD-Cltrn-/y. The activation of mTORC1 by amino acids induces phosphorylation of TFEB and remains in the cytoplasm with inactivated form. By forming mTORC1-TFEB-Rag-Regulator megacomplex[19], TFEB is critically involved in the maintenance of structural integrity of lysosomes by promoting biosynthesis of lysosomes[20] and autophagy. The phosphorylated inactive form TFEB increased in STZ-HFD-Cltrn+/y and it was reduced in STZ-HFD-Cltrn-/y associated with disappearance of giant lysosomes in the PTCs. In diabetes animal models such as C57BL/Ks db/db mice, TFEB overexpression or pharmacological activation of TFEB alleviates the tubular epithelial cell injuries by enhancing lysosomal clearance, promoting lysosomal biogenesis, and formation of autophagosomes[21, 22].

The current investigation provides the insights into drug discovery related to diabetes, obesity, and diabetic kidney disease. β amyloid precursor protein cleavage enzyme 1 (BACE1) cleaved amyloid β (Aβ) and the development of guanidine-based novel BACE1 inhibitors for the treatment and maintenance of Alzheimer's disease was attempted; however, most of the studies were discontinued[23, 24]. Collectrin is a substrate for BACE2 and it cleaves and release the collectrin ectodomain outside the cells[25]. Since transgenic mice overexpressing collectrin in pancreatic β cells resulted in increased secretion of insulin[4] and collectrin deficiency in whole body or proximal tubules causes hypertension in mice[7, 8, 26]. BACE2 inhibitor may the candidate for the treatment of type 2 diabetes and hypertension[27, 28]. However, the current study provides the evidence that the increased influx of amino acids into PTCs and activation of mTORC1 pathway may deteriorate the progression of diabetic and obesity-related kidney disease. In the model of diet-induced obesity, BACE2 knockout mice leads to

exacerbated body weight gain, hyperinsulinemia, and insulin resistance[29]. In addition, BACE2 cleaves the amyloid β protein precursor within the Aβ domain that accordingly prevents the generation of Aβ42 peptides associated with aggregation of the Aβ[30] and BACE2 loss-of-function mutation ($BACE2^{G446R}$) showed greater apoptosis and increased levels of Aβ oligomers in human pluripotent stem cell (hPSC)-derived brain organoids[31]. Further investigation is required to confirm the relevance of BACE2 inhibitor in the treatment of diabetes and obesity. In addition to BACE2, the inhibition of neutral amino acid transporter, B^0AT1 (SLC6A19), is another therapeutic approach. The inhibition of B^0AT1 activity was shown to improve the glycemic control by upregulating glucagon like peptide (GLP1) and fibroblast growth factor (FGF21) in mice[32]. B^0AT1 inhibitor is also beneficial for the diabetic kidney disease by reducing the neutral amino acids influx such as Gln and Leu and ameliorating the mTORC1 activity. Finally, the reduced phosphorylated inactive form of TFEB in STZ-HFD-Cltrn-/y suggested the beneficial effects of TFEB activator and this notion was suggested in the previous studies[21, 22].

The limitation of current investigation is that it is inconclusive whether the improvement of renal tubular lesions is due to reduced amino acid influx or systemic metabolic improvements. To further confirm the results, the investigation of the effects of altered amino acids influx on mTORC1 activity and autophagy-lysosomal function by using proximal tubule-specific *Cltrn* knockout mice or primary cultured tubular cells isolated from wild and *Cltrn* knockout mice.

Disclosures

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Author contributions

YK, KM, CK and JW designed the project and experiments and wrote the manuscript. YK, SY, KM, CK, YO, NK, RS, and HHHA performed animal experiments and analyzed and interpreted data. SY, AN and JE performed Western blotting, genotyping and other molecular biology experiments.

Data Sharing Statement

Data of *Cltrn* knockout mice (B6;129S5-*Cltrn*^{tm1Lex}Mmucd) and targeted *Cltrn* gene are available at MGI:5007377 and NM_020626.1, respectively.

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Table 1. Urinary concentrations of amino acids (mmol/mL) (unpaired t tests).

	STD-Cltrn+/y	STD-Cltrn-/y	STZ+HFD-Cltrn+/y	STZ+HFD-Cltrn-/y	STD-Cltrn+/y vs STD-Cltrn-/y	STZ+HFD-Cltrn+/y vs STZ+HFD-Cltrn-/y	STD-Cltrn+/y vs STZ+HFD-Cltrn+/y	STD-Cltrn-/y vs STZ+HFD-Cltrn-/y
	Mean ± SD				P values			
Asp	19.0 ± 7.0	208.6 ± 98.0	30.7 ± 9.3	326.2 ± 276.3	0.017682*	0.123083	0.136779	0.395936
Glu	429.7 ± 20.0	672.6 ± 202.4	382.3 ± 43.5	1016.2 ± 609.0	0.091432	0.131901	0.162092	0.265491
Asn	70.0 ± 17.3	5622.0 ± 2169.1	122.3 ± 24.4	7234.0± 5013.0	0.005135**	0.054834	0.038999*	0.527849
Ser	18.0 ± 1.0	8326.0 ± 4945.0	32.3 ± 23.5	10708.4 ± 9005.7	0.030451*	0.093964	0.349997	0.618135
Gln	26.0 ± 8.7	68240.0 ± 27578.5	101.0 ± 36.1	88500.0 ± 1493.0	0.006024**	0.011743*	0.024938*	0.389769
His	1.7 ± 2.1	4814.0 ± 1760.5	6.7 ± 5.5	7624.0 ± 3028.0	0.003754**	0.005569**	0.215285	0.110577
Gly	329.0 ± 82.5	32500.0 ± 14452.3	453.0 ± 90.1	41120.0 ± 1727.6	0.009701**	0.007551**	0.153708	0.416976
Thr	16.0 ± 10.4	18140.0 ± 5838.9	40.7 ± 4.5	19182.0 ± 11296.9	0.002004**	0.029506*	0.01983*	0.859181
Arg	19.3 ± 14.6	207.2 ± 162.4	66.7 ± 57.8	534.4 ± 610.5	0.100972	0.247159	0.241099	0.280215
Ala	337.3 ± 136.1	27740.0 ± 10444.3	676.7 ± 163.3	26240.0 ± 18731.3	0.004569**	0.062056	0.050621	0.879596
Tyr	18.0 ± 3.6	5726.0 ± 2340.2	64.3 ± 31.3	6586.0 ± 2613.8	0.006425**	0.005786**	0.063728	0.59856
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Val	27.0 ± 10.1	8582.0 ± 2425.8	47.7 ± 12.9	10148.0 ± 6363.3	0.00104**	0.037427*	0.094623	0.621012
Met	383.0 ± 59.0	3208.0 ± 1298.0	584.0 ± 125.4	4536.4 ± 3548.9	0.01073*	0.111115	0.065872	0.454476
Trp	53.7 ± 5.9	855.8 ± 357.8	87.7 ± 33.5	1310.6 ± 542.3	0.009405**	0.009196**	0.158395	0.156133
Phe	83.3 ± 8.6	2394.0 ± 797.1	131.3 ± 24.1	2830.4 ± 1793.6	0.00282**	0.045072*	0.031543*	0.632449
Ile	112.0 ± 20.0	4602.0 ± 1346.4	183.7 ± 53.9	5580.0 ± 3269.2	0.00139**	0.032507*	0.097166	0.553424
Leu	177.7 ± 26.7	10080.0 ± 2815.2	215.0 ± 45.0	11558.0 ± 7347.7	0.001054**	0.041272*	0.28433	0.685529
Lys	n.d.	993.0 ± 934.9	8.0 ± 13.9	1127.2 ± 1462.2	0.12515	0.246622	0.373901	0.867022
Pro	19.0 ± 8.2	5202.0 ± 1303.0	66.7 ± 15.0	6080.0 ± 3725.9	0.000549***	0.035265*	0.008418**	0.632302

526

FIGURE LEGENDS

Figure 1. The metabolic phenotypes of Cltrn+/y and Cltrn-/y male mice treated with standard diet (STD) or streptozotocin and high fat diet (STZ-HFD). (**A**) Body weight of Cltrn+/y (n=3) and Cltrn-/y (n=5) mice treated with STD or STZ-HFD. (**B**) Organs weight of Cltrn+/y (n=3) and Cltrn-/y (n=5) mice treated with STD or STZ-HFD at 35 weeks of age. (Epi, epididymal; Mes, mesenteric; Sub, inguinal; Brown, Brown adipose tissues) (**C**) Systolic blood pressure of Cltrn+/y (n=3) and Cltrn-/y (n=5) mice treated with STD or STZ-HFD. There are no statistical differences. Data shown as mean ± SD and analyzed by One-way ANOVA with Tukey test at each time point in **A** and **C**. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Figure 2. Glucose metabolism in Cltrn+/y and Cltrn-/y male mice treated with standard diet (STD) or streptozotocin and high fat diet (STZ-HFD). (**A**) *Ad labium* blood glucose levels in Cltrn+/y (n=3) and Cltrn-/y (n=5) mice treated with STD or STZ-HFD. (**B**) HbA1c levels in Cltrn+/y (n=3) and Cltrn-/y (n=5) mice treated with STD or STZ-HFD. (**C**) Glucose tolerance tests in Cltrn+/y (n=3) mice treated with STD or STZ-HFD and Cltrn-/y mice (STD-Cltrn-/y n=7; STZ-HFD-Cltrn-/y n=5). (**D**) Serum insulin levels during glucose tolerance tests. (STD-Cltrn+/y, n=2; STD-Cltrn-/y, n=3; STZ-HFD-Cltrn+/y, n=3; STZ-HFD-Cltrn-y, n=4). (**E**) Insulin tolerance tests. (STD-Cltrn+/y, n=4; STD-Cltrn-/y, n=3; STZ-HFD-Cltrn+/y, n=3; STZ-HFD-Cltrn+/y, n=5). Data shown as mean ± SD and analyzed by One-way ANOVA with Tukey test (**A**, **C** and **E**) and Mixed-effects analysis due to missing values (**B** and **D**) at each time point separately. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Figure 3. Respiratory quotient, oxygen consumption rate and locomotor activities in Cltrn+/y and Cltrn-/y male mice treated with standard diet (STD) or streptozotocin and high fat diet (STZ-HFD). (**A**) Daily profile of respiratory quotient in Cltrn+/y (n=3) and

552 Cltrn-/y (n=5) mice treated with STD or STZ-HFD. Significant differences between STZ-
553 HFD-Cltrn+/y and STZ-HFD-Cltrn-/y are shown. (**B**) Respiratory quotient in light and
554 dark periods. (**C**) Oxygen consumption rate in Cltrn+/y (n=3) and Cltrn-/y (n=5) mice
555 treated with STD or STZ-HFD. Significant differences between STZ-HFD-Cltrn+/y and
556 STZ-HFD-Cltrn-/y are shown. (**D**) Oxygen consumption rate in light and dark periods.
557 (**E**) Locomotor activities in Cltrn+/y (n=3) and Cltrn-/y (n=5) mice treated with STD or
558 STZ-HFD. Significant differences between STZ-HFD-Cltrn+/y and STZ-HFD-Cltrn-/y
559 are shown. (**F**) Locomotor activities in light and dark periods. Data shown as mean ±
560 SD and analyzed by Two-way ANOVA with Tukey test. (*p<0.05; **p<0.01; ***p<0.001;
561 ****p<0.0001).

562 **Figure 4**. Urinary albumin/creatinine ratio and urinary amino acids profiles in Cltrn+/y
563 and Cltrn-/y male mice treated with standard diet (STD) or streptozotocin and high fat
564 diet (STZ-HFD). (**A**) Urinary albumin/creatinine ratio (ACR) in Cltrn+/y (n=3) and Cltrn-
565 /y (n=5) mice treated with STD or STZ-HFD. (**B**) Urinary Kidney injury molecule-1
566 (Kim-1)/creatinine ratio (KCR) in Cltrn+/y (n=3) and Cltrn-/y (n=4) mice treated with
567 STD or STZ-HFD. (**C**) Concentrations of urinary amino acids in Cltrn+/y (n=3) and
568 Cltrn-/y (n=5) mice treated with STD or STZ-HFD. Data shown as mean ± SD,
569 analyzed by One-way ANOVA with Tukey test at each time point in **A** and **B**. Significant
570 differences between STZ-HFD-Cltrn+/y and STZ-HFD-Cltrn-/y are shown in **C**.
571 (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

572 **Figure 5**. PAS (Periodic Acid Schiff) stain of kidney tissues in Cltrn+/y and Cltrn-/y
573 male mice treated with standard diet (STD) or streptozotocin and high fat diet (STZ-
574 HFD). (**A** and **B**) STD-Cltrn+/y, (**C** and **D**) STD-Cltrn-/y, (**E** and **F**) STZ-HFD-Cltrn+/y,
575 and (**G** and **H**) STZ-HFD-Cltrn+/y. Red arrows in panels **E** and **F** show the vacuoles in

tubular cells. The red open squares in **A**, **C**, **E**, and **G** are areas for panel **B**, **D**, **F**, and **H**, respectively.

Figure 6. Electron micrographs of proximal tubules in Cltrn+/y and Cltrn-/y male mice treated with streptozotocin and high fat diet (STZ-HFD). (**A**) STZ-HFD-Cltrn+/y. The enlarged lysosomes (red arrows) and regular sized lysosomes (red arrowheads) are seen. The white open square is area for panel **B**. (**B**) STZ-HFD-Cltrn+/y. Various forms of autophagosomes are seen (yellow arrowheads). (**C**) The proximal tubules without giant lysosomes observed in STZ-HFD-Cltrn+/. The white open square is area for panel **D**. (**D**) STZ-HFD-Cltrn+/y. Various forms of autolysosomes (blue arrows) and lipid autolysosomes (orange arrows) are observed. (**E**) STZ-HFD-Cltrn-/y. Regular sized lysosomes (red arrowheads) are seen. The white open square is area for panel **F**. (**F**) STZ-HFD-Cltrn-/y. Autophagosomes (yellow arrowheads) and autolysosomes (blue arrows) are shown.

Figure 7. Western blot analyses in Cltrn+/y and Cltrn-/y male mice treated with standard diet (STD) or streptozotocin and high fat diet (STZ-HFD). (**A**) Western blot analyses for phospho-mTOR, mTOR, phospho-TFEB, ATG13, p62, LC3, Raptor, S6K, and GAPDH in STD-Cltrn+/y (n=4), STD-Cltrn-/y (n=5), STZ-HFD-Cltrn+/y (n=6), and STZ-HFD-Cltrn-/y (n=7) mice. (**B**) Densitometric analyses of Western blots. Data shown as mean ± SD and analyzed by Two-way ANOVA with Tukey test. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). Significant differences between STZ-HFD-Cltrn+/y and STZ-HFD-Cltrn-/y are shown.

SUPPLEMENTARY MATERIAL

Supplementary Figure S1. A. Uncropped gel images for STD groups in Figure 7A. **B.** Uncropped gel images for STZ-HFD groups in Figure 7A.