Absence of CIC5 in Knockout Mice Leads to Glycosuria, Impaired Renal Glucose Handling and Low Proximal Tubule GLUT2 Protein Expression

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Key Words

CIC-5 • Glucose homeostasis • Megalin • Proximal tubule • Glucose transporters and insulin

Abstract

Glycosuria is one of the well-documented characteristics in CIC-5 knockout (KO) mice and patients with Dent's disease. However, the underlying pathophysiology of its occurrence is unknown. In this study, we have compared CIC-5 KO mice with age and gender matched wild-type (WT) control mice to investigate if the underlying cause of manifested glycosuria is an impairment of glucose homeostasis and/or an alteration in expression levels of proximal tubule (PT) glucose transporters. We observed that, the blood glucose concentration (n=12, p<0.01) and the fractional excretion of glucose and insulin (n=6, p<0.05) were higher in KO mice. In contrast, the fasting blood glucose levels (n=7) were not significantly different in the two groups. Plasma glucose increased to a greater extent in KO mice (n=7, p<0.05) when challenged by an intraperitoneal injection of glucose. However, no peripheral tissue

insulin resistance was observed following an intraperitoneal injection of insulin (n=9) in the KO mice. ELISA analysis demonstrated low plasma insulin concentrations after a 12 hour fasting period and also following glucose injection in KO mice. The total insulin released during a 2 hour period following glucose challenge was significantly lower in KO mice (n=6, p<0.05). By western blot, we observed a significant decrease in GLUT2 protein expression levels in isolated PT ((n=10, p<0.01)) of KO mice. This decrease in protein levels was corroborated by a significant decrease in GLUT2 mRNA levels estimated semi quantitatively by RT-PCR in isolated PT (n=10, p<0.01). No significant changes in mRNA expression levels of SGLT2, SGLT1 and GLUT1, as analyzed by RT-PCR, could be detected in the isolated PT (n=10). Also, we have shown by western blot analysis that expression of megalin is lower in the renal cortex of KO mice when compared to WT mice (n=3, p<0.05). Our results suggest that low plasma insulin concentration together with renal function changes observed in KO mice significantly contribute towards the glucose intolerance and documented glycosuria observed in this animal.

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Introduction

CIC-5 is a 746-amino acid protein originally thought to belong to a family of voltage-gated chloride channels [1, 2] but more recent evidence suggests that it may function as a H^+/Cl^- exchanger [3]. In the kidney, CIC-5 is highly expressed in proximal tubules and in collecting ducts (α and β intercalated cells) [4]. In proximal tubules, CIC-5 is located at the apical endosomes together with electrogenic V-type H^+ -ATPases [4] where it has a complementary function in acidification of endosomes [5]. The physiological relevance of CIC-5 in renal function came into view when mutations in the *CICN5* gene were associated with a X-linked renal tubular disorder called Dent's disease [6, 7]. This disorder is characterized by low molecular weight (LMW) proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, aminoaciduria, phosphaturia, kaliuresis, uricosuria, glycosuria and, eventually, renal failure [8, 9]. Although many of the clinical symptoms seen in Dent's disease are variable, LMW proteinuria is consistently present and shown to be caused by an impairment in proximal tubule endocytosis [4]. The precise mechanism of this proximal tubule abnormality is not entirely clear but possibly results from defective acidification and/or a decrease in megalin content of the cells [4, 10, 11].

Although the LMW proteinuria has received much attention, the mechanism(s) underlying glycosuria in patients with Dent's disease and KO mice is not very well understood. Glycosuria is usually found in patients with hyperglycemia as seen in Diabetes Mellitus [12]. Glucose is freely filtered into the kidney at the glomerulus and under normal circumstances is completely reabsorbed in the proximal tubule [13]. Glucose is transported from the tubular lumen into the cytoplasm by the glucose transporters SGLT2 (low-affinity and high-capacity) and SGLT1 (high-affinity and low-capacity) in the brush border of the S1-S2, and S3 segments, respectively [14]. Once in the cytoplasm of tubular proximal tubule cells, glucose moves into the peritubular fluid driven by the outward chemical gradient via the basolateral glucose transporters (GLUT) such as GLUT2 (in the S1 and S2 segments) and GLUT1 (in the S3 segment) [14]. Although Diabetes Mellitus is the most common cause, genetic syndromes, such as Fanconi-Bickel syndrome caused by mutation in the facilitated glucose transporter (GLUT2) also lead to glycosuria [15].

The purpose of this work is to elucidate the possible factors responsible for development of glycosuria in CIC-5 KO mice.

Materials and Methods

Animal model

All protocol used in this work and described below were approved by the ethic committee with animal from the Johns Hopkins University. In this work, were used CIC-5 deficient mice, an animal model for Dent's disease previously published [7]. All mice used in this work were male between 12-14 weeks old weighting about 21g (WT) and 28g (KO).

Urine and plasma measurements

WT and KO mice, fed regular chow, were separately acclimatized for 72 hours in metabolic cages before collecting urine for 24 hours. After the urine has been collected a cardiac puncture was performed under deep halothane anesthesia to collect blood.

Creatinine and glucose concentrations were assessed in the urine and plasma (Anntech animal diagnostic laboratories, Baltimore, MD USA) and the clearance of both compound was calculated. Plasmatic and urinary insulin levels were determined by ELISA (Ultra Sensitive Rat Insulin ELISA Kit, CRYSTAL CHEM INC., IL, USA) for insulin clearance measurement.

The fractional excretion of glucose and insulin were calculated dividing the glucose and insulin clearance by the creatinine clearance.

Blood measurements of glucose for intraperitoneal insulin and glucose tolerance tests

A drop of tail blood was taken by making a small snip in the terminal 1.5 mm of the tail with a scalpel. Glucose concentration in the blood was determined using OneTouch[®] Ultra[®] glucose meter (Lifescan, Inc., CA, USA).

Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed as previously described [16]. Mice were fasted overnight for 12 hours. Their body weights after fasting were measured and the basal glucose levels were determined as described above ($\tau = 0$). An injection of D-glucose (Sigma, St. Louis, MO, USA) (2g/kg of fasting body weight) was performed into the peritoneum of the mice, and tail blood was taken at $\tau = 15, 30, 45, 60, 90$, and 120min post injection for glucose measurements.

Determination of plasma insulin during IPGTT

The determination of serum insulin during IPGTT was performed as previously described [16]. A snip in terminal 1.5 mm of the tail was performed with a scalpel and about 50ul of blood was collected in 1 U/ml sodium heparin using a capillary glass tube and assayed for basal insulin levels ($\tau = 0$) by the method described above. During the IPGTT, blood samples were obtained at time 15, 60, and 120min. Blood was placed immediately after collection on ice and centrifuged at $5,000 \times g$ for 5 minutes at 4°C for plasma separation. Plasma insulin levels were then assayed by ELISA. The net insulin secreted mediated by glucose was estimated by the integration of the area under the curves.

Intraperitoneal Insulin Tolerance test (IPITT)

IPITT was performed as previously described [16]. Mice were fasted for 5 hours, before the experimental procedures, to evaluate the glucose after fasting and glucose challenge. Their body weights after fasting were measured and the basal glucose levels were determined as described above ($\tau = 0$). An injection of human biosynthetic insulin (Roche, IN, USA) (0.45 unit / kg of fasting body weight) was performed into the peritoneum of the mice and the tail blood was taken at $\tau = 15, 30, 45, 60, 90$, and 120min post injection for glucose measurement.

Kidney proximal tubule isolation and total RNA extraction

Proximal tubules were isolated from WT and KO mice using the methods previously described [17]. Total RNA from proximal tubules was isolated using Trizol reagent (Gibco BRL, Grand Island, N.Y., USA).

Reverse-transcription and polymerase chain reaction

Total RNA (1 μ g) from proximal tubules was reversed transcribed using iScriptTM cDNA Synthesis Kit (BioRad, CA, USA). In the PCR reaction, cDNAs were used in the presence of 0.5 U of Taq DNA polymerase Recombinant (Invitrogen, CA, USA), 0.2 μ M of each primer, 200 μ M of each deoxynucleotide triphosphate (dNTP) (Invitrogen, CA, USA), and a commercial buffer (Invitrogen, CA, USA) containing 1.5 mM MgCl₂. Preliminary experiments were done to set the optimal conditions for the RT-PCR reactions. For RT-PCR the following mouse primers were used: I) SGLT2 primers 5'-CTT CTG TCG GGT ACA CTA CCT C-3' and 5'-CCT GGT TGT AGC TCA TGA CAT C-3' which produced a DNA segment of 497 bp, II) SGLT1 primers 5'-GGA AGC ATA TGA CCT GTT CTG-3' and 5'-GAC AGA GTG ACA TCA GAC AAC AC-3' which produced a DNA segment of 415 bp, III) GLUT2 primers 5'-GAA GAG GAG ACT GAA GGA TCT GC-3' and 5'-GTA GCA GAC ACT GCA GAA GAG C-3' which produced a DNA segment of 461 bp, IV) GLUT1 primers 5'-GGT GTT TGG CTT AGA CTC CAT C-3' and 5'-GCT CTA CAA CAA ACA GCG ACA C-3' which produced a DNA segment of 415 bp, and V) β -Actin primers 5'-TAT GCC AAC ACA GTG GTG TCT GG-3' and 5'-TAC TCC GCT GCT GAT CCA CAT-3' which produced a DNA segment of 200 bp. The identities of amplified products was confirmed by determination of molecular size on agarose gel electrophoresis (1.5% agarose in buffer containing 40 mM Tris/acetate and 1 mM EDTA) and visualized by ethidium bromide staining (0.5 μ g/ml) under ultraviolet light. The glucose transporters and β -actin bands from samples were analyzed by densitometry (Scion Image Alpha 4.0.3.2). Expression was normalized by dividing the glucose transporters values by the corresponding internal control values (β -actin).

Protein extraction and Western Blotting

The renal cortex was dissected using a Sttadie-Rigg microtome [18] and homogenized in lyses buffer [250 mM Sucrose, 20 mM HEPES, 1 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 10X ROCHE protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany) 10ng/ μ l leupeptin, 10 ng/ μ l aprotinin, 4 ng/ μ l elastatinal, 2.5 mM 1,10-phenanthroline, 100 μ M TPCK, 500

mg/ml Benzamidine, and 1 mM b-Glycerophosphate (Sigma, USA)] using a glass potter with a Teflon piston (10 strokes). After homogenization, Triton X-100 at 1% final concentration was added and the tissue homogenate was incubated on ice for 30 minutes and centrifuged at 10,000 $\times$ g for 10 minutes. The pellet was discarded and the supernatant was transferred to new tube. The proteins were solubilized in sample buffer [62.5 mM Tris-HCl pH 6.8, 2% SDS (5% SDS for megalin analyses), 5% Glycerol, 0.01% Bromophenol Blue, 1.7% β -Mercaptoethanol] and boiled for 2 minutes.

Standard SDS-PAGE was carried out, loading equal quantities of protein per lane (50 μ g) using pre-caste 10% gel (BIORAD, CA, USA) for GLUT2 and pre-caste 4-15% gradient gel (BIORAD, CA, USA) for megalin. The proteins were transferred to nitrocellulose membranes (BIORAD, CA, USA) in Tris/glycine transfer buffer (BIORAD, CA, USA). For GLUT2, the membrane was blocked with 5% BSA in PBS plus 0.1% Tween20 (PBS-T) overnight at 4°C, washed once for 10min in PBS-T and then incubated with polyclonal anti-rabbit GLUT2 antibody (Chemicon International, Temecula, CA, USA) overnight at 4°C diluted 1:2,000 in block solution. For megalin, the western blot was performed as previously described [19]. After the primary antibody incubation, the membranes were washed three times with PBS-T for 10min each wash and incubated for 1h at room temperature with horseradish peroxidase conjugated anti-rabbit antibody diluted 1:10,000. After washing for three times with PBS-T for 10min each wash, the blots were visualized using a standard ECL kit (Amersham Bioscience, Piscataway, NJ, USA). Once the GLUT2 and megalin western blots were performed, the membranes were stripped using a commercial stripping buffer (Pierce, Rockford, IL, USA) and blocked again (PBS-T plus 5% non-fat dry milk). The nitrocellulose membranes were re-probed with monoclonal anti-mouse β -Actin antibody (Chemicon International, Temecula, CA, USA) and peroxidase conjugated anti-mouse antibody, as described above.

Statistical analysis

Results are expressed as mean $\pm$ S.E. Statistical significance was determined using two tailed unpaired Student's t-test or one-way Analyses of Variance with Bonferroni's post test.

Results

Plasma glucose and fractional excretion of glucose and insulin

To begin evaluation of glucose homeostasis in the KO mice, plasma glucose and fractional excretion of glucose (FE_{gluc}) and insulin ($FE_{insulin}$) were measured (Figure 1). The blood glucose concentration of the KO mice was about 1.2 fold higher compared to WT mice (KO, 174 ± 4.33 mg/dl vs. WT, 151.18 ± 5.04 mg/dl, $n=12$, $p<0.01$, Figure 1A). Even though there were relatively small differences in plasma glucose, the FE_{gluc} was about 2.8 times higher in KO mice compared to WT mice (KO,

Fig. 1. Blood glucose and fractional excretion of glucose and insulin. *A*: The KO mice, fed regular chow, have higher blood glucose concentrations when compared to WT mice (N = 12); *B*: The fractional excretion of glucose (FE_{gluc}) in KO mice is higher compared to WT mice (N = 6). *C*: Also, the KO mice have increased fractional excretion of insulin (FE_{insulin}) compared to WT mice (N = 6). All values are represented as means ± SE. *P < 0.01 and +P < 0.05.

0.177 ± 0.04%, vs, WT, 0.064 ± 0.02%, n=6, p<0.05, Figure 1B). The most dramatic difference was seen in the fractional excretion of insulin (FE_{insulin}) which was about 15 times higher in KO mice (KO, 5.66 ± 1.54%, vs. WT, 0.38 ± 0.14%, n=6, p<0.01, Figure 1C).

These results show that the KO mice lose massive amounts of insulin in their urine and their blood glucose and FE_{gluc} are increased.

Fig. 2. Glucose and insulin tolerance tests. *A*: KO mice glucose concentrations increase dramatically following intraperitoneal glucose injection compared to WT mice (N = 7). *B*: During the intraperitoneal injection of insulin, blood glucose falls similarly in both KO and WT mice (N = 8). All values are means ± SE. *P < 0.05.

Glucose tolerance (IPGTT)

After 12 hours of fasting, the mice were challenged with an intraperitoneal injection of glucose (2g/kg of fasting body weight) to determine their glucose tolerance (Figure 2A). The resultant immediate rise in blood glucose was similar in both WT and KO mice. Fifteen minutes after glucose injection, blood glucose levels in WT mice started to decrease and after 2 hours, blood glucose levels were same as observed during the fasting period. In KO mice, there was a delay in reduction of plasma glucose levels by about 15min following glucose injection which was not observed in WT mice. In addition, 2 hours after the intraperitoneal glucose injection, blood glucose concentration of the KO mice was still significantly higher

than in WT mice (KO, 196.7 ± 22.0 mg/dl, vs. WT, 136.1 ± 9.6 mg/dl, $n=7$, $p<0.05$, Figure 2A). These results demonstrate glucose intolerance in KO mice suggesting possible impairment in glucose homeostasis.

Non-impairment of tissue glucose utilization pattern (IPITT)

After 5 hours of fasting, the KO mice blood glucose concentration was assessed and was not significantly different when compared to WT mice (KO, 146 ± 4.34 mg/dl, vs. WT, 127.67 ± 6.96 mg/dl, $n=9$, Figure 2B). After intraperitoneal injection of human biosynthetic insulin (0.45 units insulin/kg fasting body weight), blood glucose concentration was monitored for 2 hours. It is worth noting that the time course of insulin-induced reduction in blood glucose was identical in KO and WT mice ($n=9$, Figure 2B). These data show that KO mice respond normally to an insulin challenge suggesting that glucose utilization at tissue level remains unimpaired.

Plasma insulin concentrations

Twelve hours fasting plasma insulin levels were significantly lower in KO mice when compared to WT mice (KO, 0.24 ± 0.02 ng/ml, vs. WT, 0.57 ± 0.04 ng/ml, $n=6$, $p<0.01$, Figure 3A). Plasma insulin levels were also determined at 15, 60, and 120 minutes after an intraperitoneal injection of glucose. It was observed that at these time points KO mice had lower plasma insulin levels compared to WT mice (KO, 0.52 ± 0.037 , 0.59 ± 0.036 , 0.50 ± 0.039 ng/ml, vs. WT, 0.77 ± 0.053 , 0.74 ± 0.047 , 0.66 ± 0.031 , respectively, $n=6$, $p<0.01$, Figure 3A). The estimated total amount of insulin at the end of the 2 hours period following glucose challenge was significantly lower in KO mice compared to WT mice (KO - 33.99 ± 4.72 ng /ml at 120min; WT - 85.85 ± 4.49 ng/ml at 120min, $n=6$, $p<0.01$, Figure 3B).

Expression of glucose transporters

In isolated proximal tubules from WT and KO mice, mRNA expression levels of glucose transporters SGLT2, SGLT1, GLUT1 and GLUT2 were semi-quantified by RT-PCR. No significant differences in the mRNA expression levels of SGLT2, SGLT1 and GLUT1 could be detected between KO and WT mice ($n=10$, Figure 4A, C and D). The only difference was in the mRNA expression levels of GLUT2 which decreased to about $62 \pm 10\%$ ($n=10$, $p<0.01$) of the amount detected in the WT mice (Figure 4B). Similarly the GLUT2 protein expression in KO mice assayed in total cortex was estimated to be about $53 \pm 8\%$ ($n=7$, $p<0.01$) of the amount

Fig. 3. Plasma insulin concentration following intraperitoneal injection of glucose *A*: Plasma insulin concentration of KO mice after fasting and during the IPGTT is lower compared to WT mice ($N = 6$). *B*: The total glucose-mediated insulin release estimated by integration of the area under the curve is lower for KO mice compared to WT mice. All values are represented as means $\pm$ SE. * $P < 0.01$ and * $P < 0.05$.

detected in WT mice (Figure 4E). The decrease in mRNA causes decreased GLUT2 protein levels in PCT. No significant differences in expression levels of GLUT2 mRNA and protein could be detected in liver and jejunum of KO vs. WT mice (data not shown). These results suggest that decreased renal re-absorption of glucose in KO mice predominantly contribute to the documented glycosuria.

Expression of Megalin

Because megalin plays an important role in endocytosis in the proximal tubule [20] we analyzed by western blot the expression of megalin in the renal cortex. When compared to WT mice, KO mice had a much lower

Fig. 4. Expression of glucose transporters and megalin: *A, C and D:* No significant changes in mRNA expression of glucose transporters (SGLT2, SGLT1 and GLUT1) could be observed in isolated proximal tubules of KO mice compared to WT mice (N = 10). *B:* GLUT2 mRNA is lower in isolated proximal tubules of KO mice compared to WT (N = 10). *E:* GLUT2 protein expression is less in renal cortex of KO mice compared to WT (N = 7). *F:* Lower total megalin protein expression in renal cortex of KO mice compared to WT was observed (N = 3). The graphic representations are means $\pm$ SE for the ratio between densitometric values of glucose transporters or megalin by their respective β -actin. * $P < 0.01$ and * $P < 0.05$.

expression of cortical megalin protein (KO, $66 \pm 4\%$, $n=3$, $p<0.05$, Figure 4F).

Discussion

Glycosuria is defined as the appearance of glucose in urine. In presence of normal renal tubular function, all the filtered glucose is reabsorbed by the PCT [21, 22]. This process follows Tm (Transport maximum) kinetics and is saturable [23]. Glycosuria can manifest itself either secondary to increased glucose overload or failure to reabsorb normally filtered load of glucose by the PCT. On a general, scale glycosuria can be classified as Diabetic or Non Diabetic.

Glycosuria is a prominent feature of Diabetes Mellitus. It is now clear that an increased incidence of diabetes mellitus is a major health problem throughout the world [24]. Diabetes plays a central role in increasing the risk of coronary artery disease, diabetic nephropathy and other diseases [25, 26]. There are several different factors that cause Diabetes Mellitus. Type 1 Diabetes is the result of cell-mediated autoimmune attack leading to pancreatic β -cell destruction and absolute insulin deficiency [27]. Mutations in genes involved with β -cell function and insulin production are associated with a familial form of diabetes with young-age onset usually before 25 years of age that is referred to as Maturity-onset Diabetes of the Young (MODY) [28, 29]. In type 2 Diabetes the body's peripheral tissues become insulin resistant. Latter, insulin production by the pancreatic β -cells usually becomes impaired. This insulin resistance is a consequence of lifestyle and/or mutations of the genes involved in the insulin signaling [28, 30, 31].

Non Diabetic Glycosuria is an asymptomatic condition secondary to failure of glucose re-absorption despite absence of hyperglycemia, also referred to as Renal Glycosuria. A variety of genetic and toxic conditions are known to result in Non- diabetic glycosuria. Autosomal Dominant Fanconi - Bickel Syndrome (GLUT2 mutation) [32] and Familial Renal Glycosuria (SGLT2 protein mutation) [33] are some examples.

This study was undertaken to shed light on the mechanism of glycosuria in CIC-5 KO mice. In our studies we found that the fractional excretion of glucose (FE_{gluc}) is enhanced in KO mice (Figure 1B), in agreement with the glycosuria previously reported for the same animal model [7] and Dent's disease patients [8]. In light of this, we hypothesized that the Dent's disease animal model could have alterations in glucose homeostasis and/or the

glycosuria that could be aggravated by impaired renal proximal tubule glucose reabsorption. We showed that when KO mice were challenged with an injection of glucose, plasma glucose levels were slightly higher than WT (Figure 2A). This is associated with a low plasma insulin concentration during the same challenge (Figure 3A). Also, the total amount of insulin secreted at 2 hours following glucose challenge in KO mice was significantly lower compared to WT mice (Figure 3B). The tissue glucose utilization pattern demonstrated by Intraperitoneal Insulin Tolerance Test could not detect peripheral tissue insulin resistance (Figure 2B) in KO mice. With these findings we proposed that low plasma insulin concentration could be one of the factors that could contribute to the impaired glucose homeostasis observed in this animal. It is worth noting that glycosuria in KO mice occurs in spite of normal creatinine clearance / GFR [7] indicating that increased GFR is not responsible for it.

Insulin, like numerous other protein hormones and proteins, undergoes variable amount of renal processing and excretion in the PCT. It has been shown that PCT can reabsorb large amounts of insulin filtered by the glomeruli via receptor mediated endocytosis, megalin being the receptor [34]. This is a low affinity, high capacity process and responsible for the clearance of $1/3^{rd}$ of the insulin secreted by the pancreas [35]. Endocytosed insulin to a large extent undergoes high capacity lysosomal degradation at a rate directly proportional to amount of insulin endocytosed [35]. Nielsen and collaborators [36] also showed that perfused proximal tubules can carry out transtubular transport of a small portion of intact insulin from the lumen to the peritubular compartment. The same author showed in isolated proximal tubules using electron microscopy that 0.5% of the-gold marked insulin was taken-up and transcytosed to the basolateral compartment and into intercellular spaces [36]. The authors hypothesize that the transcytosis is a possible error in proteins sorting or that insulin may undergo accidental mislocation into other membrane trafficking processes.

Moreover, we showed in Figure 4F that the total amount of megalin protein is lower in the cortex of the KO mice confirming the work of others [10]. It is known that megalin is the endocytic receptor for insulin in proximal tubules cells [34]. This leads directly to the scenario that reduced megalin does not allow for enough insulin to be endocytosed by proximal tubules and ultimately decreasing its re-absorption. However we do not rule out the possibility that other changes in renal function, like increased filtration rate of insulin, could also explain the disparity between the megalin protein

expression reduction of 33% and the 12-fold increase in insulin renal excretion.

We do not exactly know the underlying mechanism for decreased plasma insulin levels. We can not reject the possibility that reduced insulin levels could be due to deficient secretion of insulin from pancreatic β -cells. It has been shown that in these cells, CIC-3 in conjunction with vacuolar type H^+ ATPase have a role in the acidification of insulin containing granules, facilitating Ca^{2+} dependent exocytosis of insulin following stimulation [37]. CIC-5 may be involved in similar functioning and insulin release but to date, there is no documented data showing that CIC-5 localizes in the pancreatic β -cells. It is also known that GLUT2 mediated uptake of glucose into β -cells determines the release of insulin from these cells [43]. It has been shown that surface GLUT2 determines the sensitivity of β -cells to plasma glucose levels [43]. There is a likely possibility that β -cells surface GLUT2 levels are decreased in KO mice, as in PCT, contributing to low plasma insulin levels.

The proximal tubule is a major site of glucose reabsorption in kidney [21, 22]. When plasma glucose is increased beyond the renal threshold, intratubular levels exceed capacity of renal proximal tubules to reabsorb all of it and glucose is excreted in final urine [13]. Although the most common reason of glycosuria is hyperglycemia, mutations in glucose transporters (such as mutations in SGLT and GLUT glucose transporters) lead to excessive loss of glucose in the urine [38-40]. In order to evaluate further the cause of glucose imbalance in KO animals we estimated expression of major glucose transporters in kidney and no differences were noted (Figure 4A, C and D) except for GLUT2. Interestingly, GLUT2 mRNA (Figure 4B) and protein (Figure 4E) expression levels were reduced in kidneys of KO mice. As showed before, high plasma glucose concentration observed in diabetic animal models can be reduced when they are treated with phlorizin or with T-1095 a synthetic phlorizin derivative (both compounds inhibit the Na^+ /glucose co-transport system in proximal tubules) [41, 42]. This treatment reduces the threshold for glucose re-absorption in proximal tubules leading to excessive loss of glucose in urine thereby, reducing the diabetic animal's plasma glucose [41, 42]. Likewise, a massive glycosuria was observed in GLUT2 mutant animals possessing a dysfunctional GLUT2 protein [15]. Thus, lower expression of GLUT2 could reduce the threshold for glucose re-absorption and it could be the reason why no major differences between KO and WT mice plasma glucose was observed in our fasted animals (Figure 2 A and B). It has previously been shown

that CIC-5 KO mice do not have hyperglycemia (accountable for the glycosuria) [7]. Interestingly, there is no documented evidence to show that patients with Dent's disease develop Diabetes Mellitus at a higher rate than general population. Here we show that there is a mild elevated plasma glucose concentration in spite of low insulin levels which can be accounted for by increased renal losses of glucose. We hypothesize that the corresponding losses of glucose prevent development of hyperglycemia, which is further aided by absence of insulin resistance (Figure 2B).

In summary, we have shown that the glycosuria in CIC-5 KO mice occurs as a result of decreased renal reabsorption of glucose in PCT and is a type of renal glycosuria. The decreased reabsorption is also associated with a reduction of GLUT2 protein expression in PCT, which possibly is one of the reasons for the decrease. Decrease in plasma insulin levels and increase in urinary excretion levels in KO mice were additional findings of this study. We showed that there is increase in the renal excretion of insulin secondary to decrease in megalin-mediated endocytosis of insulin, megalin expression being decreased in PCT of KO mice. The decrease in plasma insulin levels is still elusive. It could be secondary to decreased secretion of insulin by β cells of pancreas either due to lack of CIC5 or, more likely, due to decrease in GLUT2 in the β cells of pancreas and not just because of increased urinary loss. Further studies need to be done to uncover this mechanism. The decreased plasma insulin however seems to counter the renal loss of glucose in KO mice preventing the occurrence of hyperglycemia.

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