Medicine

A kidney-hypothalamus axis promotes compensatory glucose production in response to glycosuria

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Abstract

The kidneys facilitate energy conservation through reabsorption of nutrients including glucose. Almost all of the filtered blood glucose is reabsorbed by the kidneys. Loss of glucose in urine (glycosuria) is offset by an increase in endogenous glucose production to maintain normal energy supply in the body. How the body senses this glucose loss and consequently enhances glucose production is unclear. Using renal *Glut2* knockout mice, we demonstrate that elevated glycosuria activates the hypothalamic-pituitary-adrenal axis, which in turn drives endogenous glucose production. This phenotype was attenuated by selective afferent renal denervation, indicating the involvement of the afferent nerves in promoting the compensatory increase in glucose production. In addition, through plasma proteomics analyses we observed that acute phase proteins - which are usually involved in body's defense mechanisms against a threat – were the top candidates which were either upregulated or downregulated in renal *Glut2* KO mice. Overall, afferent renal nerves contribute to promoting endogenous glucose production in response to elevated glycosuria and loss of glucose in urine is sensed as a biological threat in mice. These findings may be useful in improving efficiency of drugs like SGLT2 inhibitors that are intended to treat hyperglycemia by enhancing glycosuria, but are met with a compensatory increase in endogenous glucose production.

eLife assessment

This study presents a **useful** characterization of mechanisms underlying glycosuria-mediated increase in compensatory glucose production in Glut2 knockout mice. The strength of support is **incomplete** but the data represent a starting point for further studies regarding the role of the HPA axis and acute phase proteins in regulating blood glucose during glycosuria.

Introduction

The kidneys help conserve energy by reabsorbing nutrients and preventing their loss in urine. Almost all of the filtered glucose is reabsorbed by the kidneys. The renal threshold for glucose reabsorption in humans is 180 mg/dl and in rodents is about 400 mg/dl[1]. In conditions such as overt hyperglycemia (beyond the renal glucose threshold), genetic loss of glucose transporters like GLUT2[2-4] or SGLT2[5], and/or renal dysfunction, glucose is excreted in urine. Enhancing glycosuria, through SGLT2 inhibition[6-8], is now used to reduce blood glucose levels in humans with diabetes. However, the glucose loss is offset by a compensatory increase in endogenous glucose production, consequently compromising the efficacy of this strategy in treatment of diabetes[9, 10]. We recently produced renal *Glut2* knockout mice[3], which show massive glycosuria and are protected from diabetes and diet-induced obesity. Despite the loss of glucose in urine, renal *Glut2* knockout mice maintain normal fasting blood glucose levels. Collectively, these observations indicate the presence of a fundamental mechanism involved in sensing glucose loss and triggering a compensatory increase in endogenous glucose production in rodents and humans. This mechanism is also aligned with the established survival strategies that reduce utilization and increase production of glucose (energy) during a flight or fight response against a threat[11, 12].

Understanding how the kidney senses glucose loss and consequently activates compensatory pathways to make up for this loss will enhance our knowledge about integrative mechanisms that regulate glucose homeostasis. Answering such research questions may also be useful in medicine to increase efficiency of drugs such as SGLT2 inhibitors that are used to reduce blood glucose levels in diabetes by elevating glycosuria.

In this study, we used renal *Glut2* knockout mice to identify mechanisms involved in activating a compensatory increase in glucose production in response to glucose loss in urine. We determined whether neural connections between the kidneys and hypothalamus can explain the compensatory increase in glucose production. In addition, we performed plasma proteomics analyses to identify secreted proteins or endocrine factors that may provide insights into pathways involved in conserving energy and producing endogenous glucose in response to elevated glycosuria in renal *Glut2* knockout mice.

Methods

Mouse husbandry

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Rochester, Yale University, or University of Iowa, and were performed according to the US Public Health Service guidelines for the humane care and use of experimental animals. Mice were housed in ventilated cages under controlled temperature (~23°C) and photoperiod (12 h light / dark cycle, lights on from 06:00 hours to 18:00 hours or 07:00 hours to 19:00 hours at Yale) conditions with free access to Hydropac water (Lab Products, USA) and regular laboratory chow (5010, LabDiet, USA or Global 2018, Harlan Teklad, USA). We used male and female renal *Glut2* knockout[3] and their littermate control mice in this study. After genotyping, mice were randomly assigned to different experimental groups. Renal *Glut2* deficiency was induced using tamoxifen as described previously[3].

Glucose production and liver metabolomics

Mice underwent surgery under isoflurane anesthesia to place a catheter in the jugular vein, and were allowed to recover for 7 days prior to flux studies. They were fasted overnight (16 h) to deplete liver glycogen prior to study. Mice were administered a 3X primed-continuous infusion of

[3-^{13}C] sodium lactate (continuous infusion rate 20 µmol/kg/min) and $^{2}H_{7}$ glucose (continuous infusion rate 2.2 µmol/kg/min) for 120 min, and euthanized with IV Euthasol. Glucose production from pyruvate was measured in vivo in the liver and kidney (both freeze-clamped in situ within 30 second of euthanasia) using mass isotopomer distribution analysis as described previously[13]. Briefly, we measured the whole-body ratio of pyruvate carboxylase flux to total glucose production by gas chromatography/mass spectrometry-mass spectrometry. We compared the whole-body gluconeogenesis from phosphoenolpyruvate (PEP) and all substrates upstream of PEP (i.e., pyruvate, lactate, amino acids) to that measured locally in the liver and kidney. As the whole-body fraction of gluconeogenesis from PEP represents an integrated contribution of both liver and kidney to whole-body glucose production from PEP, this allowed us to –calculate the fractional contribution of the kidney to whole-body glucose production.

For measuring liver metabolites involved in glycolysis, flash-frozen liver was homogenized to extract metabolites in 80% methanol, which was evaporated under liquid nitrogen followed by resuspension of metabolites in 50% methanol. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis was performed with reverse-phase LC on a Synergi Fusion-RP column (Phenomenex, Torrence CA) at 35 °C. Samples were analyzed by single reaction monitoring on a Thermo Quantum triple-quadrupole mass spectrometer. Metabolites were identified against a library of validated standards based on retention time, intact mass, collision energy, and fragment masses. Data were collected and analyzed using Thermo XCaliber 4.0 software. Post-hoc data was analyzed using Metaboanalyst 5.0. Data were median-normalized (normalized to the median peak area per sample, which correlated well with the total protein concentration). 73 metabolites were measured, out of which 9 were significantly ($p<0.05$) different (1.5 fold-change threshold, Supplementary data table 1).

Measurement of genes and hormones involved in regulating hypothalamic-pituitary-adrenal axis

We measured the levels of *Crh* mRNA in the mouse hypothalamus using RNA fluorescence in situ hybridization (*Crh* probe, 316091, ACD) according to the manufacturer's instructions. DAPI was used to stain the nucleus and verify the regions of interest in the mouse brain. Images were captured using Keyence fluorescence microscope BZ-X800. To further quantify the number of cells expressing *Crh* or DAPI, we used CellProfiler 4.4.1 (*https://cellprofiler.org/releases*).

We used the following primers to measure gene expression using RT-qPCR: *Crh*: 5'-GGAATCTCAACAGAAGTCCCGC-3' and 5'-CTGCAGCAACACGCGGAAAAAG-3', *Hrprt*: 5'-AACAAAGTCTGGCCTGTATCC-3' and 5'-CCCCAAAATGGTTAAGGTTGC-3'. All primers were used at a final concentration of 500 nmol/l. The relative quantity of each mRNA was calculated from standard curves and normalized to the internal control Hprt, and then normalized to the mean of corresponding controls.

Plasma levels of adrenocorticotropic hormone (ab263880, Abcam) and corticosterone (80556, Crystal Chem) were measured using ELISA per the manufacturers' instructions.

Ablation of afferent renal nerve

After anesthetizing mice using 1 – 5% isoflurane, a dorsal midline incision was made to access both the kidneys. We selectively ablated afferent renal nerves using capsaicin. Gauze, soaked in capsaicin (35 mM in 10% ethanol and 90% saline solution), was wrapped around the renal blood vessels for 15 min. Other surrounding tissues were covered with parafilm to avoid their exposure to capsaicin. After the surgery, muscle layers and skin were closed with nylon sutures. This procedure ablates afferent renal nerves without affecting the blood vessels or efferent renal nerves[14]. Control mice underwent the same procedure without capsaicin (sham surgery using 10% ethanol and 90% saline solution). The mice were allowed to recover for a week before determining the effects of the afferent denervation on blood glucose and other parameters.

Measurement of renal nerve activity, blood pressure, and heart rate

The mouse renal nerve activity was measured using multifiber recording as described previously [15]. After anesthesia, the nerve innervating the left kidney was identified, dissected free, and placed on a bipolar 36-gauge platinum–iridium electrode (A-M Systems; Carlsborg, WA). The electrode was connected to a high impedance probe (HIP-511; Grass Instruments Co., Quincy, MA), and the nerve signal was amplified 10^5 times with a Grass P5 AC pre-amplifier and filtered at low and high frequency cutoffs of 100 Hz and 1000 Hz, respectively. This nerve signal was directed to a speaker system and to an oscilloscope (54501A, Hewlett–Packard Co., Palo Alto, CA) for auditory and visual monitoring of the nerve activity. The signal was then directed to a resetting voltage integrator (B600C, University of Iowa Bioengineering) that sums the total voltage output in units of 1 V * sec before resetting to zero and counting the number of spikes per second. The final neurograms were continuously routed to a MacLab analogue–digital converter (8S, AD Instruments Castle Hill, New South Wales, Australia) for permanent recording and data analysis on a Macintosh computer. Renal sympathetic nerve activity was measured during 30 min. To measure the afferent renal nerve activity, the nerve was cut distally and the signal was recorded for 30 min. Nerve activity was corrected for post-mortem background activity to eliminate background electrical noise in the measurements.

In addition to the nerve activity, arterial pressure and heart rate were measured using a tapered MRE-040 tubing inserted into the left carotid artery and the other end connected to a transducer (BP-100; iWorks System, Inc., Dover, NH) that led to an ETH-250 Bridge/Bio Amplifier (CB Sciences; Milford, MA). Core body temperature of the mouse was measured using a rectal probe (YSI 4000A Precision Thermometer; Yellow Springs, OH) and maintained constant at 37.5 °C using a custom-made heated surgical platform.

Two-dimensional difference gel electrophoresis (2D-DIGE)

Plasma proteins from control and renal *Glut2* knockout mice were labeled with Cy3 and Cy5 dyes respectively. We also used an internal standard (labeled with Cy2 dye) containing equal amounts of protein of each plasma sample as a quality control. Using 2D gel electrophoresis, we separated plasma proteins (30 µg in 2D sample buffer) on a gel by both isoelectric point and molecular weight. We then imaged the gel using Typhoon scanner. DeCyder software was used to identify protein spots of interest (downregulated or upregulated by at least 25%), which were automatically picked from the 2D gel with Ettan Spot Picker (Amersham BioSciences). The proteins were identified by MALDI TOF/TOF mass spectrometry (AB SCIEX TOF/TOF™ 5800 System) using Swiss-Prot database.

Statistics

Data are shown as mean ± s.e.m. Results were analyzed by two-tailed Student's unpaired t-test or two-way ANOVA followed by a Tukey's post hoc multiple comparison test when appropriate. All analyses were performed using Prism version 8.0.1 (GraphPad, USA) and differences were considered statistically significant at $p<0.05$.

Results and Discussion

Increased hepatic and renal glucose production in renal *Glut2* knockout mice

Renal *Glut2* knockout mice exhibit normal fasting blood glucose levels despite massive glycosuria[3]. This observation suggests a compensatory increase in endogenous glucose production, similar to that observed in humans following SGLT2 inhibition[9, 10, 16].

Therefore, we measured hepatic and renal glucose production in renal *Glut2* knockout and their littermate control mice. We observed that both tissues contribute to the compensatory increase in glucose production in renal *Glut2* knockout mice (**Figure 1a-c**). This was accompanied by a decrease in hepatic glucose 6-phosphate, glucose 1-phosphate, and fructose 6-phosphate (**Figure 1d-f**), suggesting a decline in glucose metabolism in favor of increasing glucose production. List of all measured metabolites is included in Supplementary data table 1.

Renal *Glut2* knockout mice have an activated hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis is a major stress response system involved in enhancing glucose production. Therefore, we measured plasma corticosterone and adrenocorticotropin hormone coupled with gene expression of hypothalamic corticotropic releasing hormone (*Crh*). We observed an increase in all these factors (**Figure 2**) that comprise the HPA axis. These findings suggest that the HPA axis may contribute to the compensatory increase in glucose production in renal *Glut2* knockout mice.

Afferent renal nerves contribute to enhancing glucose production in renal *Glut2* knockout mice

To determine the role of afferent renal nerves in triggering a compensatory glucose production in response to glycosuria, we used capsaicin to selectively suppress afferent renal nerve activity in renal *Glut2* knockout mice. Afferent denervation was confirmed by measuring renal pelvic CGRP (pg/mg protein) at the end of this study (Sham: 86.3 ±6.4 (control mice), 93.7 ±8.2 (renal *Glut2* knockout mice); Capsaicin: 4.7 ±0.06 (control mice), 6.4 ±0.7 (renal *Glut2* knockout mice), using ELISA kit, Cayman Chemical, 589001). Mice were allowed to recover for a week before we fasted them to measure their blood glucose levels. Because reinnervation may occur following the denervation, we completed this study within 14 days after the denervation procedure. We observed that fasting and fed (random) blood glucose levels were about 50% decreased in renal *Glut2* knockout mice compared to their control littermates (**Figure 3a,b**) after the afferent renal denervation. In addition, the denervation reversed the activation of the HPA axis in renal *Glut2* knockout mice (**Figure 3c,d**). Fasting plasma insulin levels were not changed (**Figure 3e**) after the denervation. These observations suggest that renal nerves contribute to the compensatory increase in glucose production.

Although the afferent renal denervation attenuated the compensatory increase in glucose production in renal *Glut2* knockout mice, there was no change in their total or afferent renal nerve activity (Supplementary Figure 1a,b). The *Glut2* knockout mice also had similar blood pressure, heart rate coupled with similar weights of brown and white adipose tissue, kidney, and liver (Supplementary Figure 1c-j). These findings suggest the role of integrative neuroendocrine mechanisms independently of changes in nerve activity, fat, kidney, or liver mass, in triggering a compensatory increase in glucose production in renal *Glut2* knockout mice. Overall, the results indicate that afferent renal nerves contribute to a compensatory increase in glucose production to make up for glucose loss in renal *Glut2* knockout mice. These findings support data from humans[16] that renal nerves may be involved in mediating a compensatory increase in glucose production in response to elevated glycosuria by SGLT2 inhibition.

Changes in circulating acute phase proteins in renal *Glut2* knockout mice

To further investigate underlying mechanisms compensating for glucose loss in *Glut2* knockout mice, we measured levels of secreted proteins that may explain the increase in endogenous glucose production. We performed 2D-DIGE followed by proteomic analyses with plasma samples

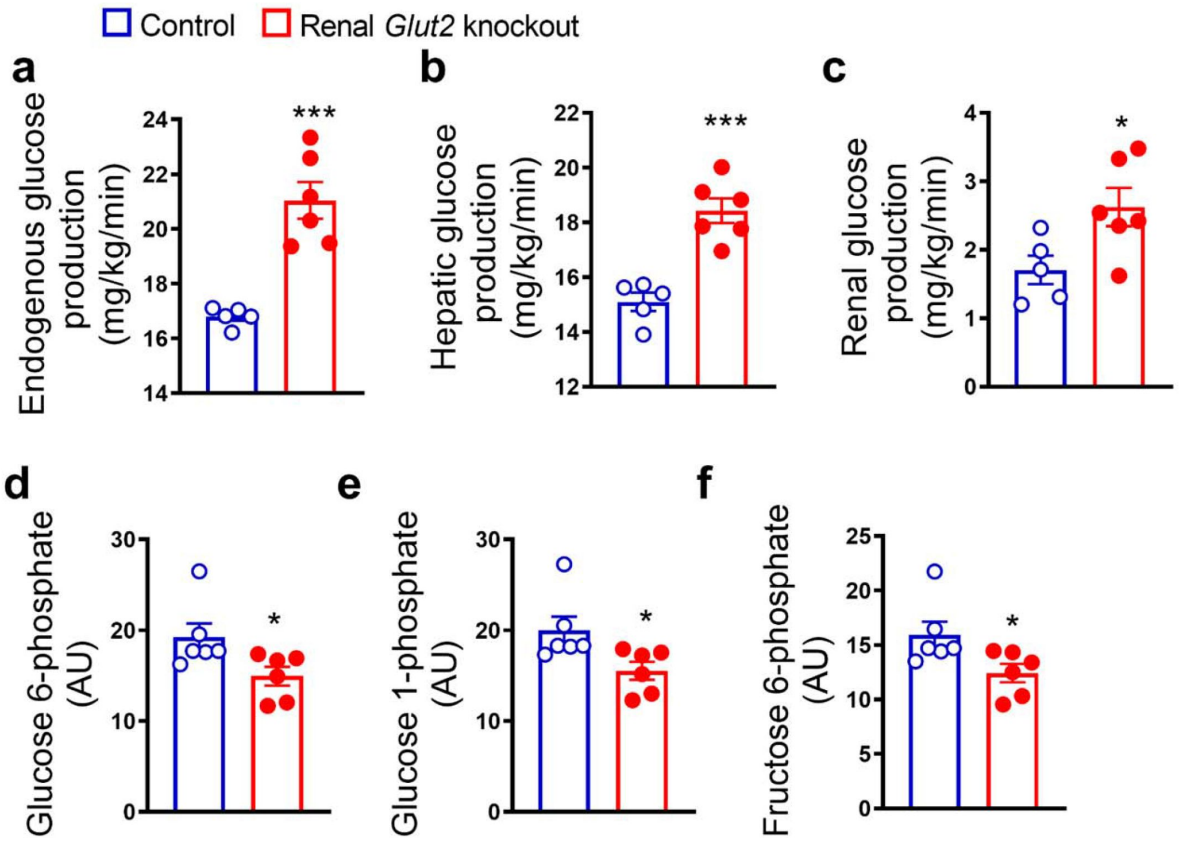

Figure 1.

Renal *Glut2* knockout mice exhibit increased glucose production. In vivo increase in total **(a)**, hepatic **(b)**, and renal **(c)** glucose production through gluconeogenesis with pyruvate as a substrate in 28 weeks old male renal *Glut2* knockout mice. Decreased hepatic glucose 6-phosphate **(d)**, glucose 1-phosphate **(e)**, and fructose 6-phosphate **(f)** in renal *Glut2* knockout mice. *p<0.05, ***p<0.001, unpaired 2-tailed Student's t-test. Data are presented as mean ±sem.

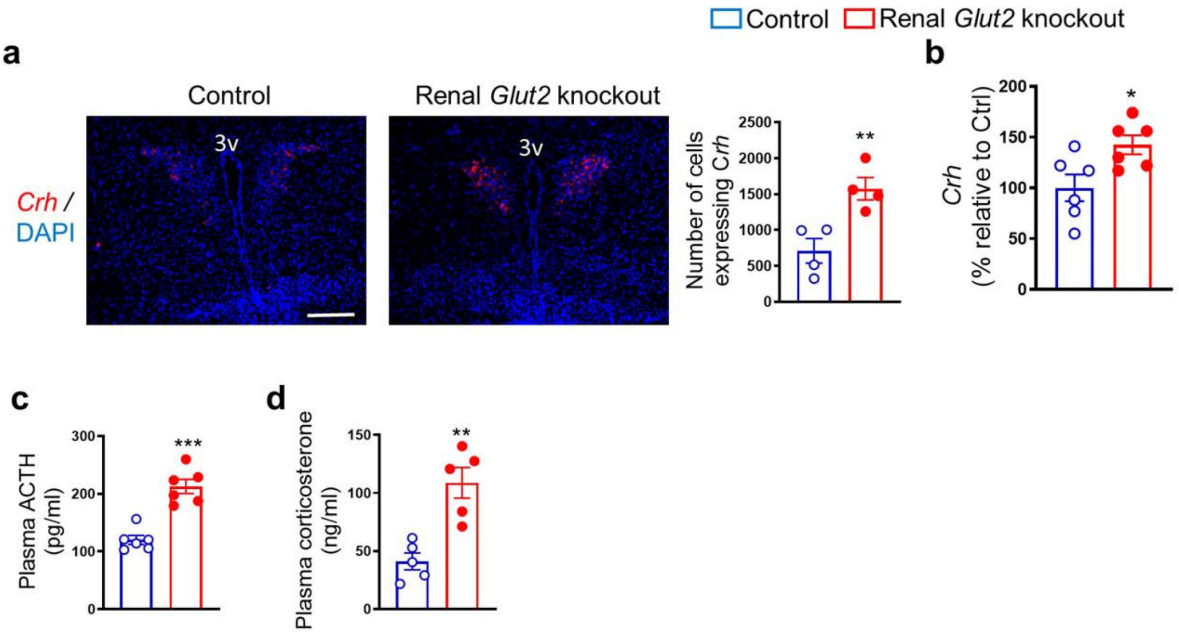

Figure 2.

Enhanced activity of hypothalamic-pituitary-adrenal axis in renal *Glut2* knockout mice. Representative images from fluorescence RNA in situ hybridization showing an increase in expression of corticotropin releasing hormone (Crh) in the paraventricular nucleus of the hypothalamus in 28 weeks old male renal *Glut2* knockout mice **(a)**. Scale, 100 µm. For the quantification shown next to the images, 4 sections per mouse and 3 areas of interest per section were analyzed in 4 mice. qRT-PCR analysis showing an increase in hypothalamic *Crh* **(b)**, data from ELISA demonstrating an increase in plasma adrenocorticotropic hormone (ACTH) **(c)** and corticosterone **(d)** in 12 weeks old male renal *Glut2* knockout mice. *p<0.05, **p<0.01, ***p<0.001, unpaired 2-tailed Student's t-test. Data are presented as mean ±sem.

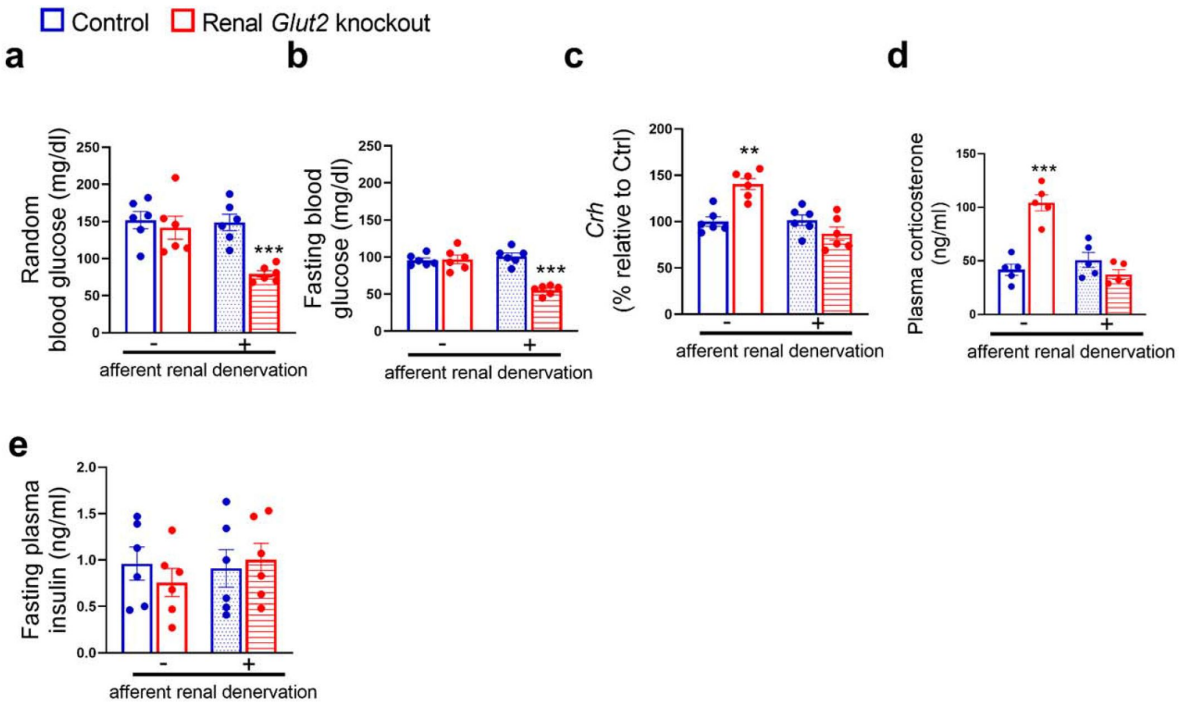

Figure 3.

Effects of afferent renal denervation on blood glucose levels and hypothalamic-pituitary-adrenal axis in renal *Glut2* knockout mice. Afferent renal denervation decreases fed (random) and fasting (overnight, 6:00 pm – 9:00 am) blood glucose levels **(a**,**b)**, restores expression of hypothalamic corticotropin releasing hormone (Crh) **(c)** measured using RT-qPCR, and plasma corticosterone **(d)** without affecting plasma insulin levels **(e)** in 30 weeks old female renal *Glut2* knockout mice. **p<0.01, ***p<0.001, two-way ANOVA followed by a Tukey's post hoc multiple comparison test. Data are presented as mean ±sem.

collected from renal *Glut2* knockout mice and their littermate controls. We observed that acute phase proteins like mannose binding lectin, albumin, haptoglobin, ferritin were either upregulated or downregulated in renal *Glut2* knockout mice (**Figure 4** and Supplementary table 2). Usually levels of these proteins are changed in response to threat, infection, and/or injury to activate body's defense systems[17]. Similarly, major urinary proteins (MUP) like MUP18, which belong to the lipocalin protein family, were elevated in renal *Glut2* knockout mice (**Figure 4** and Supplementary table 2). These proteins are involved in regulating inter-organ chemical signaling, pheromonal communication, and energy metabolism[18]. We also observed that secreted glutathione peroxidase 3 (Gpx3) was the most downregulated protein in plasma in renal *Glut2* knockout mice (**Figure 4** and Supplementary table 2). Gpx3 is predominantly expressed in the kidneys and is an anti-oxidant[19]. We validated these findings using qRT-PCR. Renal *Gpx3* gene expression was about 50% lower (Supplementary Figure 2a) in renal *Glut2* knockout mice compared to their littermate controls. Moreover, renal - but not hepatic - *Mup18* gene expression was higher (Supplementary Figure 2b,c) in renal *Glut2* knockout mice relative to their controls, suggesting that the kidneys were responsible for increasing plasma MUP18 in the knockout mice. Altogether, these results suggest that multiple pathways - including local (renal) stress and general (HPA axis) stress response combined with the acute phase proteins - compensate for glucose loss and defend against a perceived biological threat in renal *Glut2* knockout mice.

This study has limitations. Higher urine volume (polyuria) observed in renal *Glut2* knockout mice may contribute to increase in glucose production to some extent, however that contribution would likely be minor[20, 21] based on previous studies about the role of diuretics (which increase urine volume) in glucose production. The extent to which the changes in circulating secretory proteins observed in renal *Glut2* knockout mice contribute to their compensatory increase in glucose production is unknown. Further studies are necessary to address the role of these secretory proteins in glucose and energy homeostasis. It is possible that afferent renal denervation in the present study attenuated only hepatic glucose production through the hypothalamus without affecting the compensatory increase in renal (local) glucose production. If validated, this would explain the source of remaining endogenous glucose after the denervation in renal *Glut2* knockout mice.

In summary, we report that a kidney-hypothalamus axis contributes to triggering a compensatory increase in endogenous glucose production in response to elevated glycosuria in mice. Glucose loss in urine appears to be sensed as a biological threat and therefore activates body's defense systems including some of the acute phase proteins. These findings may explain why SGLT2 inhibitors don't achieve their full potential in lowering blood glucose levels during treatment of diabetes mellitus.

Author contributions

TSF designed and performed experiments including mouse genotyping, analysed results, prepared graphs and figures, and edited the manuscript. XZ and RJP measured glucose production, analysed results, and edited the manuscript. DAM and KR performed electrophysiological recordings, analysed the results, and edited the manuscript. JR and SB performed microscopy, qRT-PCR or ELISA, and edited the manuscript. PB performed metabolomics, analysed the results, and edited the manuscript. KHC conceived the study, designed and performed experiments, analysed results, wrote and edited the manuscript. All authors approved the final version of the manuscript.

Disclosures

Authors declare they have no conflict of interests.

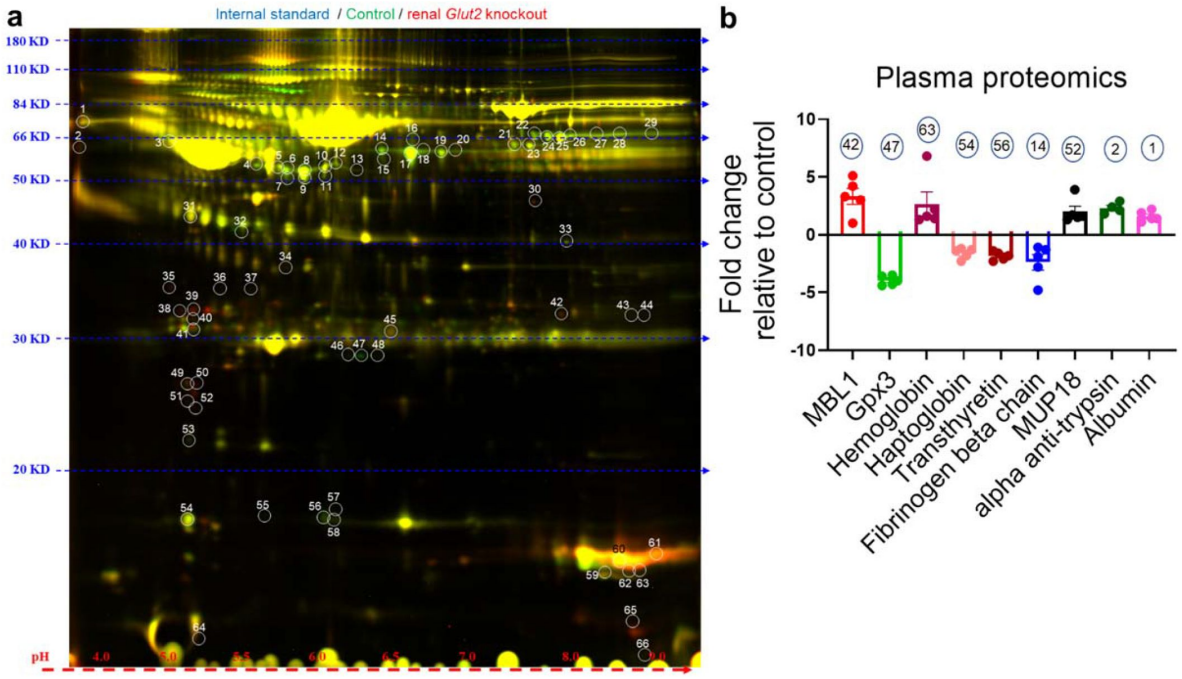

Figure 4.

Changes in levels of plasma proteins in renal *Glut2* knockout mice. Representative image of two-dimensional difference gel electrophoresis with numbered protein spots of interest is shown in **(a)**. Internal standard was prepared using equal amounts of protein of each plasma sample as a quality control (Cy2 labeled, pseudo blue), plasma proteins from control group were labeled using Cy3 dye (shown in pseudo green), and plasma proteins from renal *Glut2* knockout mice were labeled using Cy5 dye (shown in pseudo red). The identified proteins and their fold change in 28 weeks old male renal *Glut2* knockout mice compared to the control group is shown in **(b)**. The number on each bar graph in **(b)** represents the corresponding protein spot on the gel shown in **(a)**. MBL1, mannose binding lectin 1; Gpx3, glutathione peroxidase 3; MUP18, major urinary protein 18.

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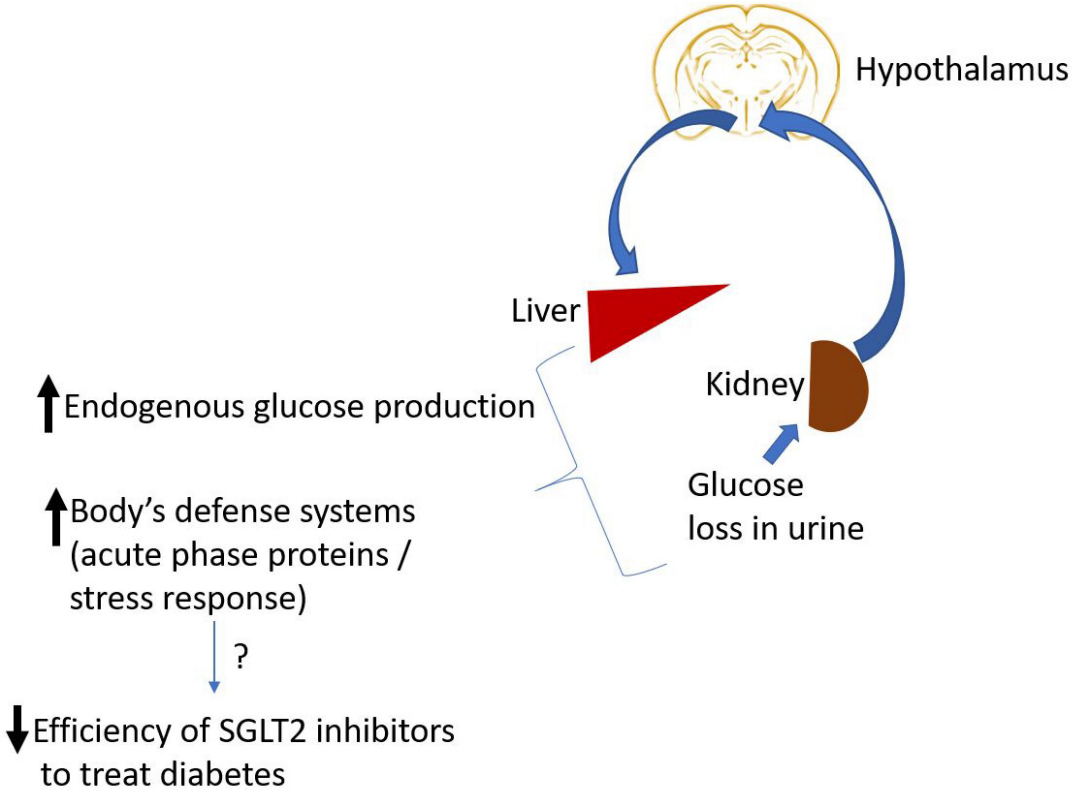
Data and materials availability

All data are available in the main text or the supplemental materials. The reagents and mouse model used in this study are available via material transfer agreement addressed to the corresponding author.



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Reviewer #1 (Public Review):

Summary:
In this study, Faniyan and colleagues build on their recent finding that renal Glut2 knockout mice display normal fasting blood glucose levels despite massive glucosuria. Renal Glut2 knockout mice were found to exhibit increased endogenous glucose production along with decreased hepatic metabolites associated with glucose metabolism. Crh mRNA levels were higher in the hypothalamus while circulating ACTH and corticosterone were elevated in this model. While these mice were able to maintain normal fasting glucose levels, ablating afferent renal signals to the brain resulted in substantially lower blood glucose levels compared to wildtype mice. In addition, the higher CRH and higher corticosterone levels of the knockout mice were lost following this denervation. Finally, acute phase proteins were altered, plasma Gpx3 was lower, and major urinary protein MUP18 and its gene expression were higher in renal Glut2 knockout mice. Overall, the main conclusion that afferent signaling from the kidney is required for renal glut2 dependent increases in endogenous glucose production is well supported by these findings.

Strengths:
An important strength of the paper is the novelty of the identification of kidney-to-brain

communication as being important for glucose homeostasis. Previous studies had focused on other functions of the kidney modulated by or modulating brain activity. This work is likely to promote interest in CNS pathways that respond to afferent renal signals and the response of the HPA axis to glucosuria. Additional strengths of this paper stem from the use of incisive techniques. Specifically, the authors use isotope-enabled measurement of endogenous glucose production by GC-MS/MS, capsaicin ablation of afferent renal nerves, and multifiber recording from the renal nerve. The authors also paid excellent attention to rigor in the design and performance of these studies. For example, they used appropriate surgical controls, confirmed denervation through renal pelvic CGRP measurement, and avoided the confounding effects of nerve regrowth over time. These factors strengthen confidence in their results. Finally, humans with glucose transporter mutations and those being treated with SGLT2 inhibitors show a compensatory increase in endogenous glucose production. Therefore, this study strengthens the case for using renal Glut2 knockout mice as a model for understanding the physiology of these patients.

Weaknesses:
A few weaknesses exist. Clarification of some aspects of the experimental design would improve the manuscript. However, most concerns relate to the interpretation of this study's findings. The authors state that loss of glucose in urine is sensed as a biological threat based on the HPA axis activation seen in this mouse model. This interpretation is understandable but speculative. Importantly, whether stress hormones mediate the increase in endogenous glucose production in this model and in humans with altered glucose transporter function remains to be demonstrated conclusively. For example, the paper found several other circulating and local factors that could be causal. In addition, the approach used in these studies cannot definitively determine whether renal glucose production or only hepatic glucose production was altered. This model is also unable to shed light on how elevated stress hormones might interact with insulin resistance, which is known to increase endogenous glucose production. That issue is of substantial clinical relevance for patients with T2D and metabolic disease. Finally, while findings from the Glut2 knockout mice are of scientific interest, it should be noted that the Glut2 receptor is critical to the function of pancreatic islets and as such is not a good candidate for pharmacological targeting.

Reviewer #2 (Public Review):

Summary:
The authors previously generated renal Glut2 knockout mice, which have high levels of glycosuria but normal fasting glucose. They use this as an opportunity to investigate how compensatory mechanisms are engaged in response to glycosuria. They show that renal and hepatic glucose production, but not metabolism, is elevated in renal Glut2 male mice. They show that renal Glut2 male mice have elevated Crh mRNA in the hypothalamus and elevated plasma levels of ACTH and corticosterone. They also show that temporary denervation of renal nerves leads to a decrease in fasting and fed blood glucose levels in female renal Glut2 mice, but not control mice. Finally, they perform plasma proteomics in male mice to identify plasma proteins with a greater than 25% (up or down) between the knockouts and controls.

Strengths:
The question that is trying to be addressed is clinically important: enhancing glycosuria is a current treatment for diabetes, but is limited in efficacy because of compensatory increases in glucose production.
Also, the mouse line used is an inducible knockout, thus minimizing the impact of compensatory mechanisms engaged in early development.

Weaknesses:

1. Though the Methods specify that both male and female mice were used, it appears each experiment was performed only on one sex, rather than each experiment being performed on both sexes. For example, renal denervation was performed only on females, whereas all other experiments were performed exclusively on males. This makes it impossible to examine whether there are sex differences in any measures.

2. This study appears to use an inducible Glut2 knockout with tamoxifen, yet nothing describes when the tamoxifen was delivered relative to the experimental manipulations. Was the knockout performed in young animals? In adult animals? This is important both for the ability of readers to repeat the experiment, but also to interpret the results in light of potential compensatory changes (if the knockout was performed at an early age, for example).

3. In Methods, please clarify whether littermate controls were WT, het, or both. If het mice were used as controls, this is potentially problematic.

4. Conclusions like "the HPA axis may contribute to the compensatory increase in glucose production in renal Glut2 knockout mice" (line 215) are premature. All that is shown is that renal Glut2 male mice have elevated HPA activity. There are no experiments establishing causation. For example, the authors could administer a CRF antagonist or a glucocorticoid receptor antagonist in this mouse line, and examine whether this impacts blood glucose. This was not done.

5. If elevated glycosuria drives HPA activity, one would expect to see elevated HPA activity in humans who take SGLT2 inhibitors. Yet, this does not seem to be the case (Higashikawa et al, 2021; see also Perry et al, 2021 for rodent example). This raises the question of whether the glycosuria observed in the mouse line here is relevant to any human conditions. The relevance of the mechanisms proposed here would be much more convincing if a second model of glycosuria was used here (for example, inducing diabetes in mice and treating with SGLT2 inhibitors). Without these types of experiments, any relevance to human conditions is highly speculative and should be reserved for the Discussion. What the authors are studying here is one mechanism for maintaining blood glucose when glycosuria is induced by a genetic knockout.

6. The experiment examining the impact of renal denervation is nice but incomplete. For example, what is the relevance to the hepatic glucose production that was reported? It is interesting that the renal denervation normalized the elevated HPA activity in Glut2 female mice, but it is not clear how this signaling would alter HPA activity.

7. The Methods need to describe the plasma collection procedure for both ELISA and plasma proteomic experiments. What time of day were samples collected? Were samples collected when animals were euthanized from other experiments after experimental manipulations, or in animals without other experimentation?

8. In general, the links between the disparate mechanisms (signals in the plasma, changes in renal activity, changes in HPA activity) are weak. There are more experiments needed to establish a direct kidney-hypothalamus axis. If renal activity elevates blood glucose in the face of glycosuria, why are there no differences in renal activity between control and Glut2 knockout mice? If the blood glucose levels are regulated by renal activity, it must be the sensitivity to the renal activity that differs between control and knockout mice - perhaps this should be investigated. If one stimulates afferent renal nerves, can one drive HPA activation and elevate blood glucose? How are these measures related to the plasma proteins identified? Without these links, this study is descriptive and correlational.

Author Response

We appreciate the reviewers' and editors' advice on further improving this manuscript. We have provided point by point responses to the reviewers' comments mentioned below. A revised version of this manuscript will be uploaded within a few weeks.

Authors' response to Reviewer 1 comments:

• We appreciate the reviewer's time in highlighting the strengths and weaknesses of this manuscript.

• Per the reviewer's advice, we will provide further description of the methods in a revised version of this manuscript.

• The interpretation about the biological threat in response to elevated glycosuria in renal Glut2 KO mice is based on our observation that these mice exhibit changes in acute phase proteins measured using plasma proteomics. We will further discuss this in a revised version of this manuscript.

• We acknowledge that this manuscript provides a resource for future mechanistic studies. Because multiple secreted proteins are changed between the control and experimental groups, some of them could be causal and others corelative in the context of enhancing compensatory glucose production in response to elevated glycosuria. Through future studies we will determine the causal proteins that trigger the increase in glucose production and identify the tissues that secrete these proteins.

• We have shown previously (Cordeiro et al., Diabetologia 2022) that renal Glut2 deficiency doesn't change insulin sensitivity (i.e. renal Glut2 KO mice don't exhibit insulin resistance despite the activation of the HPA axis). It is likely that the massive glycosuria in renal Glut2 KO mice may overcome or mask the phenotype of insulin resistance potentially induced by an increase in the stress hormones.

• In this manuscript, our major goal was to determine how elevated glycosuria leads to an increase in compensatory glucose production. We are not suggesting renal Glut2 as a therapeutic in this manuscript (that was already demonstrated in our previously published manuscript, Cordeiro et al., Diabetologia 2022).

Authors' response to Reviewer 2 comments:

1. Renal Glut2 KO mice didn't exhibit sex differences for the variables reported in our previous manuscript (Cordeiro et al., Diabetologia 2022). Therefore, in the present manuscript we decided to use male or female mice depending on their availability for each reported experiment. Per the reviewer's advice, we will describe these details including age and sexes in each figure legend.

2. For the method description, we have cited previous publications and mentioned 'as described previously'. Based on the reviewer's suggestion we will further describe the methods in detail to clarify the reviewer's concerns. In addition, we will include age and sexes in the legends of each figure.

3. For littermate controls, we had used Glut2loxp/loxp mice (which are like WT controls as described in Cordeiro et al., Diabetologia 2022) that were injected with tamoxifen exactly in the same way as the experimental mice. Het mice for Cre were not used as controls because they would have confounded the results as pointed out by the reviewer.

4. Because elevated HPA activity is known to increase blood glucose levels, we suggested 'the HPA axis may…..'. Given the nature of this manuscript, we agree the secreted proteins identified using plasma proteomics could contribute to enhanced glucose production directly or through secondary mechanisms. Afferent renal denervation using capsaicin reduced blood glucose levels concomitant with the suppression of the HPA axis in renal Glut2 KO mice. Based on these findings we speculated that the HPA axis may be partly responsible for increasing glucose production in renal Glut2 KO mice.

We had considered using CRF antagonist and glucocorticoid receptor antagonists to determine the causal role of the HPA axis in contributing to the increase in glucose production in renal Glut2 KO mice. However, these drugs activate compensatory mechanisms including changes in insulin sensitivity. Therefore, use of these drugs would further confound the results instead of providing a clarity on the causal role of the HPA axis in enhancing glucose production in renal Glut2 KO mice.

1. We understand the reviewer's concerns whether the results reported here are translatable to humans. Please note that expression of SGLT2 is not kidney-specific; therefore, pleiotropic effects of SGLT2 inhibition in tissues other than the kidney cannot be excluded in animal models and humans. In contrast, the mouse model reported in this manuscript is kidney-specific Glut2 KO mice. Therefore, phenotype produced in renal Glut2 KO mice cannot be directly compared with that produced after SGLT2 inhibition. It may be too early to speculate whether the results reported in this manuscript are translatable to humans.

In the referred research papers by the reviewer, the authors have used either models of different types of diabetes or included individuals with diabetes in their study. Notedly, diabetes itself affects the HPA axis independently of SGLT2 or GLUT2 inhibition. Therefore, it may not be appropriate to compare results obtained from animals or individuals with diabetes with that reported in this manuscript from renal Glut2 KO mice.

1. Yes, we are currently performing mechanistic studies including assessment of mitochondrial function in renal Glut2 KO mice to determine whether and how the kidneys sense loss of glucose in urine.
2. We apologize for the lack of methods description. We will provide additional method details in a revised version of this manuscript. All the assays were performed as per manufacturer's instructions. Aliquots of the same samples were used for analyses of the hormones and for consistency across different assays.