Diabetic Complications Consortium
Final Progress Report
October 13, 2022

Application Title: HP-MRI organ imaging to identify the risk of DKA with SGLT2 inhibitors in DM.

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1. Project Accomplishments:

The major goal of this project was to develop non-invasive method based on hyperpolarized (HP)-MRI to assess the potential of SGLT2 inhibitors to cause diabetic ketoacidosis (DKA) (Aim 1). This is a moderately common but inconsistent complication of the use of these increasingly important therapeutic tools. We had developed recently with colleagues in Radiology an HP-MRI-based method for comparing liver and renal metabolism, particularly gluconeogenesis (Morze CV, et al. **Sci Rep**, 8:2088, 2018), and subsequent to that, approaches to detecting mitochondrial activity and ketone body metabolism (von Morze C, et al. **Magn Reson Med**, 2018; 79:1862-1869). Our secondary goal (Aim 2) was to confirm, complement and extend beyond the HP-MRI data using approaches based on traditional biochemistry in genetically modified mice and cutured cells.

Prior to the onset of the pandemic, we started the HP-MRI work (Aim 1) (see below), and gathered preliminary data, which was accepted for presentation at the national meeting of the Am Soc Nephrology (ASN), and published in abstract form in J Am Society of Nephrol (2021). We demonstrated that we could detect markers of GNG and effects of the SGLT2 inhibitor, dapagliflozin (dapa) on renal and hepatic metabolism. This promising result was directly relevant to our planned non-invasive in vivo characterization of the effects of dapa on both GNG and ketogenesis, and hence DKA.

Unfortunately, due to the pandemic, the animal research arm of the HP-MRI facility closed entirely for several months during 2020-2021 and once reopened was on a limited schedule. During that time, a key personnel (Dr. Cornelius von Morze, a K-awardee co-mentored by me and Dr. Dan Vigneron) left UCSF to join the faculty at Washington University. Dr. von Morze was the principal scientist on the HP-MRI "tech" side of the project. Consequently, the HP-MRI work was further disrupted, and we focused on Aim 2 and had two additional abstracts accepted for presentation at the ASN and published in J Am Soc Nephrol. (see below). Currently, there is one project-related paper under review, and another in preparation.

2. Specific Aims

Specific Aim 1: HP-MRI image ketone bodies turn over and gluconeogenesis (GNG) in real time *in vivo* with rats with T1 and T2DM treated with SGLT2 Inhibitors.

Results:

Pilot study: 6 healthy sprague dawley rats aged 6-8 week were purchased from Charles River, Wilmington, MA. The animals were randomly divided into two groups, three for dapa treatment and three for vehicle control. The animals were fasted overnight (16h). Dapa 5mg/kg was injected intraperitoneally (IP). Blood glucose was measured from tail tip with a hand-held whole-blood glucometer every 15 min for the first hour followed by every 30 min

for a second hour (Figure 1). A transient effect of dapa to lower blood glucose was found. We further performed initial pilot experiments for HP-MRI, We then went on to perform HP-MRI experiments as described **(1)**. The study is described in the following abstract accepted for presentation at the ASN meeting

Hyperpolarized MRI detection of Dapagliflozin effect on Gluconeogenesis in live animals: *proof of principle*

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Introduction: SGLT2 inhibitors including dapagliflozin (dapa) ameliorate hyperglycemia by inducing glucosuria but also induce renal gluconeogenesis (GNG), thus blunting its efficacy to some extent. The lack of insight into the relative roles of kidney and liver in different states is due at least in part to limitations in the technology to separately assess liver and kidney GNG in live animals. Our study exploits a powerful technology, Hyperpolarized Magnetic Resonance Imaging (HP-MRI), which can detect metabolic conversions non-invasively in specific organs in animals and humans, in real time, in vivo. Notably, the stable isotopes of carbon (13-C) used here are approved for multiple diagnostic uses in humans, for example in monitoring prostate and hepatic metabolism in cancer.

Methods: metabolic features of healthy WT (male, age ~12 weeks) rats were studied *in vivo* using hyperpolarized (HP) ^{13}C magnetic resonance imaging (MRI), a powerful new imaging modality for non-invasive metabolic investigations, based on ~50,000-fold nuclear magnetic resonance (NMR) signal enhancements of ^{13}C-labeled substrates via dissolution dynamic nuclear polarization (DNP). We interpreted our results in comparison with results of [1- ^{13}C] pyruvate tolerance test (PTT) analysis.

Results: We successfully, detected the dapa-mediated conversion of [1-^{13}C]pyruvate to [1-^{13}C]lactate and [1-^{13}C]alanine in the liver and kidneys of rats. We found that Intravenously injected HP [1- ^{13}C]pyruvate was rapidly metabolized to [1- ^{13}C]lactate and [1- ^{13}C]alanine in the liver and kidneys of rats. The PTT data show that there is a clear trend toward increase in blood glucose following [1- ^{13}C]pyruvate injection (fig HP-MRI peaks and summary ….).

Conclusion: We establish here that HP-MRI technology can detect SGLT2i effects on metabolism in live rats, and is likely to be feasible in humans. We propose that it could be useful in characterizing sub-categories of T2DM, and in better investigating risk factors for SGLT2i-induced euglycemic ketoacidosis.

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Roadblocks: The project was progressing extremely well, however, the HP-MRI facility was closed during much of 2021 due to the COVID-19 pandemic. When ultimately reopened, they had refocused priorities due in part to a key personnel (Dr. von Morze) leaving UCSF.

We redirected our efforts toward Specific Aim 2, which did not involve HP-MRI.

Specific Aim 2: Biochemical investigation of test candidate mediators for their role in the association of SGLT2 inhibitors with DKA.

With the curtailment of access to HP-MRI, we focused on in vitro and in vivo physiological and biochemical characterization of SGLT2, both regulation by mTORC2 and inhibition by dapa.
We published the below abstract in JASN and presented at the ASN national meeting.

We made significant progress in two areas:

1. Cell Culture Characterization of role of mTORC2 in regulating SGLT2 using CRISPR-Cas9.

mTORC2 is essential for Sodium-Glucose co Transporter 2 Function

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Introduction: The role of mammalian target of rapamycin (mTOR) complexes mTORC1 and mTORC2 ion transport in kidney tubule has been well characterized. We and other have shown that mTORC2 is the hydrophobic motif kinase of serum and glucocorticoid kinase 1 (SGK1) and that its activity is required for epithelial Na^+ channel (ENaC)-dependent sodium reabsorption in the aldosterone-sensitive distal nephron (ASDN). In contrast the role of mTOR in the renal proximal tubule cell (RPTC) transporters remain obscure. It has been shown that mTORC1 activity is increased in diabetes, in RPTCs which was prevented by the inhibition of sodium-glucose co transporter 2 (SGLT2), indicating a connection between mTOR and SGLT 2. In this study we found that knock out of mTORC2 in the HEK-293Tcells inhibited the SGLT2 sodium current in the whole cell patch clamp assays.

Methods: We used CRISPR-Cas9 to generate Sin1 (an essential component of mTORC2)-deficient HEK-293T cells, which were compared with wild type cells. The cells were transiently transfected with SGLT2. Dapagliflozin-sensitive whole cell SGLT2 sodium current was measured. We recorded in WT HEK-293T cells the Dapa-sensitive SGLT2 sodium current. Strikingly, in mTORC2-knockout HEK-293T cells the Dapa-sensitive SGLT2 sodium current was significantly reduced versus WT HEK-293T cells.

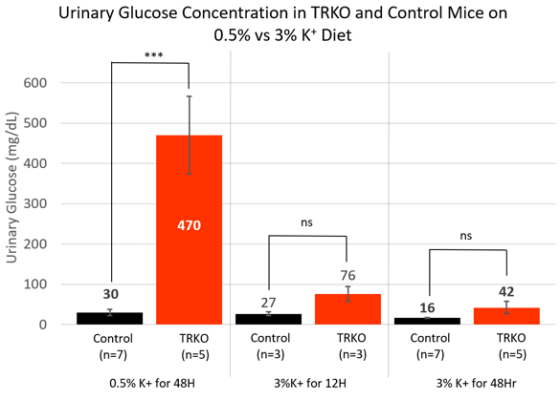
Conclusion: Knockout of mTORC2 in the HEK-293T cells inhibits SGLT2-sodium current. Our study delineates the essential role of mTORC2 in SGLT2 function. These observations may explain the broad role of SGLT2 inhibition therapy, observed in cardioprotective effect including various types of hypertension.

Acknowledgements: Financial support for this work provided by the NIDDK Diabetic Complications Consortium (RRID:SCR_001415, www.diacomp.org), grants DK076169 and DK115255' to WS and NIH RO1-DK 56695 to DP

2. In vivo studies on renal glucose handling in mTORC2 KO mice. These studies provided important preliminary data for a successful competing renewal of R01-DK56695, and was accepted for presentation at the ASN annual meeting.

Background: Insulin signaling promotes proximal tubule glucose transport and suppresses gluconeogenesis (GNG). An important feature of GNG in the proximal tubule is its dual regulation by insulin and pH. The kinase mTORC2 is well known to be regulated by insulin signaling in multiple cell types, but its mechanistic role in proximal tubule glucose homeostasis is unknown.
Methods: Rictor is a critical component of the mTORC2 complex. We generated tubule-specific Rictor KO mice (TRKO) using the doxycycline inducible Pax8-Cre Rictor$^{fl/fl}$. Mice were adapted to 1% K^+ diet and then switched to either a 0.5% K^+ or 3% K^+ diet for 2 days. An additional cohort of mice were maintained on a 0.5% K^+ diet for 2 weeks for glucose (1g/kg), insulin (0.75U/kg), and pyruvate (2g/kg) tolerance testing after overnight fasts. Renal function, serum glucose, and urine glucose were measured in metabolic cages during the last 24 hours of all experiments. Target proteins were measured via Western blot from whole kidneys.
Results: TRKO mice on a 0.5% K^+ diet had glycosuria, however TRKO mice switched to a 3% K^+ diet for 2 days had resolution of glycosuria (Figure 1). TRKO mice on a 3% K^+ diet also developed hyperkalemia and elevated BUN.

A) Effect of K+ on glycosuria in WT and TRKO mice.



B) Blood glucose is not sginficantly different between WT and TRKO mice on normal K+ diet.

Serum Parameters in TRKO and Control Mice under Normal K (0.5%) Diet for 48 Hours			
	Control (n=7)	TRKO (n=5)	
Na (mmol/L)	141	141	0.6890
K (mmol/L)	4.8	4.9	0.5343
Cl (mmol/L)	116	112	0.0395
iCa (mmol/L)	1.05	1.09	0.5115
TCO2 (mmol/L)	20	23	0.0618
Glu (mg/dL)	298	319	0.4751
BUN (mg/dL)	25	28	0.3425
Cr (mg/dL)	<0.2	<0.2	NA
Hb (g/dL)	13.0	13.2	0.5715

B) Blood glucose is not sginificantly different between WT and TRKO mice on high K+ diet.

Serum Parameters in TRKO and Control Mice under High K (3%) Diet for 48 Hours			
	Control (n=6)	TRKO (n=6)	p
Na (mmol/L)	144	136	0.0025
K (mmol/L)	4.8	7.2	0.0121
Cl (mmol/L)	115	116	0.6228
iCa (mmol/L)	1.20	1.02	0.0165
TCO2 (mmol/L)	23	18	0.0573
Glu (mg/dL)	279	253	0.2054
BUN (mg/dL)	15	37	0.0079
Cr (mg/dL)	<0.2	<0.2	NA
Hb (g/dL)	12.8	15.7	0.0246

We also found that there were no differences in serum glucose during glucose and insulin tolerance tests between groups at any timepoint. Serum glucose during intraperitoneal pyruvate tolerance test was higher in TRKO mice compared to controls at 90 (149 vs 194mg/dL; $p<0.01$) and 120 minutes (150 vs 182mg/dL; $p<0.05$) by t-test with Bonferroni correction. There was no difference in plasma membrane SGLT2 and GLUT2 abundance between TRKO and control mice after 2 days on either 0.5% or 3% K^+ diets. There were also no differences in PEPCK abundance or plasma membrane SGLT2 and GLUT2 abundance between TRKO and control mice after 2 weeks on a 0.5% K^+ diet.

Conclusions: This study demonstrates the importance of mTORC2 in glucose handling and metabolism by the renal tubules. Increased urine glucose may reflect an abnormality in glucose transporters or abnormal GNG. Blood glucose is not significantly different, which likely reflects compensatory effects of liver. The resolution of glycosuria in TRKO mice on a 3% K^+ diet could be due to mTORC2-independent suppression of GNG by K^+ but this will require further investigation. Future studies will focus on identifying the molecular defect causing increased glycosuria in TRKO mice, and the basis for glycosuria suppression by K^+. It is uncertain if abnormal SGLT2 activity plays a role in this process.

3. Publications:

No peer reviewed papers have been published. The project has resulted in:

1. Three abstracts accepted for presentation at Kidney Week of the American Society of Nephrology, and published in J Am Soc Nephrology (two in 2021 and one in 2022):
    a. Hyperpolarized MRI Detection of Dapagliflozin Effect on Gluconeogenesis in Live Animals: Proof of Principle. Waheed Shabbir, Enzo Takagi, Michael Ohliger, David Pearce. J Am Soc Nephrol 32, 2021.
    b. mTORC2 Is Essential for Sodium-Glucose Cotransporter 2 Waheed Shabbir, John E. Demko, Enzo Takagi, Bidisha Saha, Deise C. Leite- Dellova, Bharathi Sambandam, David Pearce. J Am Soc Nephrol 32, 2021
    c. Glycosuria in Tubule-specific mTORC2 Knockout Mice Resolves on a High Potassium Diet John Demko, Bidisha Saha, Enzo Takagi, Anna Manis, David Pearce. J Am Soc Nephrol, in press, 2022.
2. One paper under review and one in preparation:
    a. Potassium selectively activates mTORC2-dependent SGK1 phosphorylation to stimulate ENaC. Bidisha Saha, Waheed Shabbir, Enzo Takagi, Xin-Peng Duan, Deise Carla Almeida Leite Dellova, John Demko, Anna Mannis, Dominique Loffing-Cueni,Johannes Loffing, Wen-Hui Wang, Mads Vaarby Sørensen, and David Pearce. Under review.
    b. Glycosuria in Tubule-specific mTORC2 Knockout Mice Resolves on a High Potassium Diet John Demko, Bidisha Saha, Enzo Takagi, Anna Manis, David Pearce. In preparation.