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Direct cardiac actions of Ertugliflozin

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DIRECT CARDIAC ACTIONS OF ERTUGLIFLOZIN

by

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ABSTRACT

Sodium-Glucose Linked Transporter 2 (SGLT2) inhibitors block renal glucose reabsorption and have shown marked cardiac protection in type 2 diabetics, and surprisingly, also in non-diabetics. However, the mechanism by which these drugs improve cardiovascular outcomes is unknown. Metabolic heart disease, which is characterized by cardiac hypertrophy and diastolic dysfunction, is associated with obesity and insulin resistance and leads to adverse cardiovascular outcomes including heart failure with a preserved ejection fraction. A high fat, high sucrose "Western" diet can induce metabolic syndrome, an aggregate of obesity-driven clinical phenotypes including insulin resistance, elevated triglycerides, hypertension, and abnormal cholesterol. Using a mouse model of metabolic syndrome and adult rat ventricular myocytes (ARVMs) *in vitro*, we aim to determine if the SGLT2 inhibitor Ertugliflozin (ERTU) can prevent metabolic syndrome-induced cardiac pathophysiology and whether ERTU can exert a direct action on cardiomyocytes, a cell type lacking SGLT2. SGLT2 inhibitors have been proposed to act directly on the Sodium-Hydrogen Exchanger 1 (NHE1) and thus could have direct action on cardiomyocytes that may mediate cardioprotective effects.

Mice were fed either a control diet (CD) or a high fat high sucrose (HFHS) diet ± ERTU for 16 weeks. Echocardiography was performed and heart weights were obtained. ARVMs were used to assess ERTU's effect on insulin sensitivity *in vitro* in a high-palmitate, insulin resistance model, and to test the efficacy of the known NHE1 inhibitor

Cariporide (NHEi). A NHE1 activity ammonium chloride pulse assay was performed in HEK293 cells over-expressing either wild-type (WT) or a known NHEi-insensitive point mutant NHE1 ± NHEi or ERTU.

In HFHS-fed mice, ERTU attenuated weight gain and restored blood glucose, insulin, hemoglobin A1c, and HOMA-IR to CD levels. HFHS-induced cardiac hypertrophy and diastolic dysfunction were prevented with ERTU. *In vitro*, high palmitate media decreased insulin stimulated AKT signaling compared to low palmitate media and was rescued by either ERTU or NHEi treatment. ERTU inhibited WT NHE1 activity in HEK293 cells by 67%, whereas activity of the NHEi-insensitive mutant NHE1 was unaffected by ERTU treatment.

ERTU prevented the hallmarks of diet-induced metabolic heart disease (cardiac hypertrophy and diastolic dysfunction) in mice. These benefits exceed the expected consequences of glucose control alone. The actions of ERTU on ARVMs *in vitro* suggest the favorable effects on cardiac structure and function may be due, at least in part, to the direct action of the drug on cardiomyocytes. Furthermore, mutational overexpression studies show that ERTU can directly affect NHE1 in cardiac myocytes. Taken together, this thesis provides evidence that the direct cardioprotective effects of ERTU could be via inhibition of NHE1, a critical modulator of intracellular pH and sodium in the cardiomyocyte, with known implications in the pathophysiology of diabetes and heart failure.

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LIST OF ABBREVIATIONS

ARVM ..... Adult Rat Ventricular Myocyte

BSA ..... Bovine Serum Albumin

CD ..... Control Diet

DMEM ..... Dulbecco's Modified Eagle Media

Em ..... Myocardial Peak Early Diastolic Velocity

ERTU ..... Ertugliflozin

HbA1c ..... Hemoglobin A1c

HFHS ..... High Fat High Sucrose

HFpEF ..... Heart Failure with Preserved Ejection Fraction

HFrEF ..... Heart Failure with Reduced Ejection Fraction

HOMA-IR ..... Homeostatic Model Assessment of Insulin Resistance

HP ..... High Palmitate

KH ..... Krebs Henseleit

LP ..... Low Palmitate

NHE1 ..... Sodium-Hydrogen Exchanger 1

NHEi ..... Sodium-Hydrogen Exchanger inhibitor (Cariporide)

pH_i ..... Intracellular pH

SGLT2 ..... Sodium-Glucose Linked Transporter 2

WT ..... Wild-Type

INTRODUCTION

Metabolic Syndrome and Type 2 Diabetes

Metabolic syndrome is a collection of clinical characteristics including obesity, elevated triglycerides, insulin resistance/type 2 diabetes, hypertension, and abnormal cholesterol levels[1]. Together, the components of metabolic syndrome promote progression to cardiovascular disease[2, 3], with obesity and insulin resistance reported as crucial determinants[4].

Type 2 diabetes is dramatically increasing in prevalence both in North America and around the world. As of 2017, 46 million Americans and 425 people worldwide had type 2 diabetes, numbers that are projected to increase by 35-38% by 2045[5]. Furthermore, diabetic patients have a 2-fold increased risk for developing heart disease and increased mortality due to heart disease compared to non-diabetics[2, 6]. The metabolic syndrome-associated risk of heart disease is amplified by findings that certain anti-diabetic therapies enhance morbidity and mortality[7, 8]. However, tight regulation of glycemia is ineffective at reducing cardiovascular mortality in diabetics[9, 10]. Thus, patients with metabolic syndrome present the need for novel therapies that safely treat both diabetes and cardiovascular disease in conjunction.

Metabolic Heart Disease and Heart Failure

Metabolic heart disease, a cardiomyopathy common in patients with obesity-related metabolic syndrome, is characterized by left ventricular hypertrophy and diastolic

dysfunction[1, 11]. Metabolic heart disease is one of the most common causes of heart failure with a preserved ejection fraction (HFpEF)[12, 13]. HFpEF is a growing epidemiological problem, accounting for nearly half of all hospital admissions for heart failure[14, 15]. No treatment thus far has clearly demonstrated benefits on outcomes in HFpEF patients, in contrast to several FDA-approved therapies that exist for heart failure with reduced ejection fraction (HFrEF). Thus, there is the unmet need for viable therapies for HFpEF patients who often have concurrent metabolic syndrome.

Sodium-Glucose Linked Transporter 2 Inhibitors

Located in the renal proximal tubule, Sodium-Glucose Linked Transporter 2 (SGLT2) is responsible for 90% of glucose reabsorption. Inhibition of SGLT2 leads to a 10-fold increase in urine glucose, as well as modest reductions in hemoglobin A1c, body weight, and blood pressure[16]. SGLT also transports sodium, so that its inhibition causes sodium excretion in the urine. The first SGLT2 inhibitor to be FDA approved for type 2 diabetes treatment was Empagliflozin. The subsequent EMPA-REG OUTCOME study was conducted in accordance with FDA requirements to specifically evaluate cardiovascular risk in the type 2 diabetic patient population. Surprisingly, Empagliflozin treatment conferred dramatic cardiovascular protection, with a 38% decreased risk of cardiovascular death, 32% decreased risk of death from any cause, and 35% decreased risk of hospitalization for heart failure[17]. Other trials of SGLT2 inhibitor class members have yielded similar findings[18, 19], and it appeared to occur regardless of diabetic status[20].

In light of these striking observations, it is unclear if SGLT2 inhibitors exert direct action on the heart [8, 21–23]. Although initially the leading hypothesis of SGLT2 inhibitor efficacy was attributed to systemic effects of SGLT2 inhibition (e.g. a diuretic effect), focus has turned to if and how SGLT2 inhibition may be affecting direct cardiac remodeling given the paucity of improvement seen with diuretics. Moreover, off-target effects have been hypothesized to play a role considering the heart does not express SGLT2[24–26].

Sodium-Hydrogen Exchanger 1

Comprised of a family of 9 isoforms, Sodium-Hydrogen Exchanger 1 (NHE1) is prevalent on cardiomyocyte sarcolemma and responsible for intracellular pH regulation via sodium import and hydrogen (H^+) export. NHE1 abundance and activity have previously been reported to increase in both type 2 diabetes and heart failure pathophysiology[27]. Importantly, enhanced NHE1 activity has been implicated in cardiac hypertrophy[28, 29] and insulin resistance[30].

Baartscheer *et al* was the first group to show that Empagliflozin inhibited NHE1 flux in rabbit cardiomyocytes, as measured by intracellular pH and sodium changes during an ammonium pulse[31]. Furthermore, they showed that pretreatment with Cariporide (NHEi), a specific NHE1 inhibitor, ablated any effect of Empagliflozin, suggesting Empagliflozin acts via NHE1[31]. In a follow-up study, Uthman *et al* found that two other SGLT2 inhibitors, Dapagliflozin and Canagliflozin, can also inhibit NHE1 flux in freshly isolated mouse cardiomyocytes, indicating a class effect of SGLT2 inhibitors[32].

Additionally, binding affinity for all three SGLT2 inhibitors in the proposed sodium-binding pocket of NHE1 was implicated by simulated docking studies[32]. Taken together, there is mounting evidence of glucose-independent effects of SGLT2 inhibitors, plausibly via blockade of NHE1, although to date simulated docking studies are the most concrete evidence of direct action.

Aims

We have previously shown that mice fed a high fat, high sucrose (HFHS) diet develop obesity and type 2 diabetes as well as metabolic heart disease hallmarks left ventricular hypertrophy and diastolic dysfunction[33], mimicking disease progression in patients with metabolic syndrome[11, 34, 35]. However, it is unknown if SGLT2 inhibitors can protect hearts in this model, and more specifically if SGLT2 inhibitors can elicit cardioprotective effects on insulin-resistant cardiomyocytes directly. The goals of this thesis research are to 1) test whether the SGLT2 inhibitor, Ertugliflozin (ERTU), exerts beneficial cardiac effects in a mouse model of metabolic heart disease, 2) test whether ERTU exerts direct effects *in vitro* using freshly isolated primary adult rat cardiomyocytes treated with high palmitate to induce insulin resistance, and 3) test whether the *in vitro* effect of ERTU is mediated via NHE1 by mutating the known NHEi binding site.

METHODS

Chemicals were purchased from Sigma-Aldrich unless otherwise stated. The animal protocol was approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Experimental Animals

Six-week-old male C57BL/6J mice were purchased from Jackson Laboratory, housed four per cage, and allowed to adjust to the Boston University animal facility in conventional cages with house chow diet for two weeks prior to initiation of the study. At eight weeks of age, cages were randomized by average body weight using random.org random number generator to either control diet (CD, 10% kcal lard, 0% sucrose, D09071703 Research Diets), CD with Ertugliflozin (ERTU, 0.5 mg/g of diet, Merck), high fat high sucrose diet (HFHS, 58% kcal lard, 28% sucrose, D09071702 Research Diets), or HFHS with ERTU, n=25 per group (see Table 1 for diet compositions). Mice were fed for 4 months, during which body weights and food consumption were recorded weekly.

Diet Type :	CD		ERTU CD		HFHS		ERTU HFHS	
Composition	***gm%***	***kcal%***	***gm%***	***kcal%***	***gm%***	***kcal%***	***gm%***	***kcal%***
Protein	15	15	15	15	20.5	15	20.5	15
Carbohydrate	76.3	75	76.3	75	38.2	28	38.2	28
Fat	4.5	10	4.5	10	35.5	58	35.5	58
Total (%)		**100**		**100**		**100**		**100**
Total (kcal/gm)	**4.05**		**4.05**		**5.54**		**5.54**	
Ingredient	***gm***	***kcal***	***gm***	***kcal***	***gm***	***kcal***	***gm***	***kcal***
Casein, 80 Mesh	182	728	182	728	182	728	182	728
DL-Methionine	3	12	3	12	3	12	3	12
Maltodextrin 10	170	680	170	680	170	680	170	680
Corn starch	760	3040	760	3040	0	0	0	0
Sucrose	0	0	0	0	164	656	164	656
Lard	55	495	55	495	320	2880	320	2880
Salt Mix, S10026B	50	0	50	0	50	0	50	0
Vitamin Mix, V10001	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0
Ertugliflozin	**0**	**0**	**0.616**	**0**	**0**	**0**	**0.451**	**0**
Ertugliflozin (mg/gm diet)	**0**		**0.5**		**0**		**0.5**	
Total	**1232**	**4995**	**1232.6**	**4995**	**901**	**4996**	**901.45**	**4996**

Table 1: Diet Compositions. CD, ERTU CD, HFHS, and ERTU HFHS diet component macromolecule breakdown and ingredients. Diets sourced and compounded at Research Diets, Inc.

Fasting Blood Measurements

Prior to blood collection, mice were fasted overnight. Blood glucose was measured by a ContourNext glucometer (Bayer) and hemoglobin A1c (Crystal Chem) was determined per manufacturer's instructions. In addition, blood was collected via tail vein into heparinized microvettes (Sarstedt), centrifuged at 2000g for 5 minutes to separate plasma, and plasma was further centrifuged at maximum speed for 15 minutes to deplete platelets from the plasma. Plasma insulin levels were measured via Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem) according to manufacturer's directions. The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index was calculated using the formula [fasting blood glucose (mg/dL) × fasting plasma insulin (μU/mL)] ÷ 405.

Echocardiography

Tissue doppler echocardiography was measured with a VisualSonics Vevo 2100 high-resolution imaging system equipped with a 22-5-MHz MS550D transducer, as previously described[33]. Mice were anesthetized via isoflurane, and the apical view four-chamber of the heart was used to record myocardial tissue movement at the mitral annulus. Myocardial peak early diastolic velocity (Em) was measured via this method over 5 consecutive cardiac cycles, averaged, and analyzed via Vevo 2100 Analytic software.

Organ Weights

At the conclusion of 16 weeks, mice were euthanized via isoflurane inhalation within a bell jar until non-reactive to a toe pinch, followed by rapid excision of the heart. Left ventricles were dissected and weighed. One leg was excised above the knee and subjected to x-ray for tibia length measurement. Concurrent with the x-ray a size marker (such as a penny) was placed in the image field. ImageJ was used to determine the tibia lengths of each mouse in millimeters. Left ventricle weights were normalized to tibia length as an index to body size.

Adult Rat Ventricular Myocyte Isolation

Adult Rat Ventricular Myocytes (ARVMs) were isolated from 150-200g male Sprague Dawley rats, as previously described[36]. In brief, rats were anesthetized with

isoflurane in a bell jar until non-reactive to a toe pinch, the heart was rapidly excised and cannulated by the aorta onto a retrograde perfusion system (Radnoti MasterFlex), and perfused with 37°C Krebs Henseleit (KH) buffer containing (in mM): 118 NaCl, 4.75 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 25 $NaHCO_3$, pH 7.4, for the initial 2 minutes of perfusion. The perfusate was then switched to KH containing collagenase type II (Worthington) and hyaluronidase, which was allowed to recirculate for 16 minutes. The heart was removed from the cannula, ventricles minced, and incubated in KH containing collagenase, hyaluronidase, DNAse, trypsin, and 1 mM $CaCl_2$ in a shaking water bath for 20 minutes. The ventricular slurry was pipetted up and down to further dissociate cells, then passed through a 100 μm filter. Cells were centrifuged at 500 rpm for 3 minutes, supernatant removed, and the ventricular myocyte pellet was resuspended in a wash buffer comprised of equal parts KH and Dulbecco's Modified Eagle Media (DMEM 12320-032, Life Technologies). Cells were allowed to settle by gravity for 10 minutes, supernatant removed, and resuspended in the wash buffer. This step was repeated, and upon final resuspension, cells were layered atop a 5% Bovine Serum Albumin (BSA) in DMEM gradient and allowed to settle for 11 minutes. The resulting pellet comprised primarily viable cardiomyocytes, which were resuspended and plated onto laminin-coated dishes. After 30-60 minutes, media was changed by gentle aspiration to remove unattached and non-viable cells, and experiment-specific media and treatments were added to the plates (described in next section).

Adult Rat Ventricular Myocyte Culture and Treatment Conditions

Palmitate Media Preparation: Palmitic acid (200 mM) was initially dissolved in 200-proof ethanol, then diluted to 5 mM in KH buffer. 1.2 molar excess KOH was added to the solution and was heated at 70°C for 15 minutes (vortexing every 5 minutes) to saponify the palmitic acid. Saponified palmitic acid was added stepwise to pre-warmed (≤40°C) DMEM containing fatty acid free BSA (65.6 μM) and low glucose (5.5 mM) for a final concentration of 200 μM, or a 3:1 molar ratio of palmitic acid:albumin ("High Palmitate"). "Low Palmitate" media was made following the same steps, but starting with 0.5 mM in KH buffer, for a final concentration of 20 μM, or 0.3:1 molar ratio of palmitic acid:albumin. Medias were further supplemented with 2 mM carnitine, 5 mM creatine, 5 mM taurine, and penicillin/streptomycin, then sterile filtered and stored at -80°C in aliquots until ready to use.

Insulin Resistance Model: 30-60 minutes after initial plating of freshly isolated ARVMs, media was changed to either Low Palmitate (LP) or High Palmitate (HP) media. Simultaneous to palmitate media application, Ertugliflozin (ERTU, 200 ng/mL = 0.353 μM) or the sodium hydrogen exchanger 1 inhibitor Cariporide (NHEi, 10 μM final concentration). The concentration of ERTU was chosen based on physiological plasma levels of ERTU in patient cohorts, which range from 112.9 to 360.8 ng/mL [37, 38]. NHEi concentration was based on manufacturer's recommendations and previous studies[31, 32]. Vehicle controls PBS or DMSO were used for ERTU or NHEi treatments, respectively. After 24 hours, media was fully changed and treatments re-applied. At 48 hours, cells

were stimulated with insulin (100 nM final concentration) for 15 minutes, then rinsed with PBS, and scraped in 1% SDS Lysis buffer (1x PBS, 5 mM EDTA, 1 mM vanadate).

SDS-PAGE and Immunoblotting

Cell lysates were sonicated until no longer viscous, and protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce). Normalized lysates were subjected to SDS-PAGE on mini-protean 10% TGX gels (Bio-Rad), transferred to PVDF membranes. PDVF was blocked for 30 minutes with Li-Cor Odyssey blocking buffer, then primary antibodies were incubated overnight at 4°C. Primary antibodies used were mouse-α-AKT (Cell Signaling #2920) and rabbit-α-phospho-AKT (Cell Signaling #4060). Blots were washed 3x with TBS with tween 20 (0.1%), incubated in Li-Cor secondary antibodies for 60 minutes, washed further 3x, then imaged on the Li-Cor Odyssey near-infrared scanner. Densitometry analyses were performed using the Li-Cor Odyssey software.

Site-directed Mutagenesis of NHE1

pYN4+, an expression plasmid containing a HA-tagged human full length NHE1 cDNA sequence, was a gift from Larry Fliegel (Addgene plasmid # 78715; http://n2t.net/addgene:78715; RRID:Addgene_78715)[39]. Previous reports describe Phenylalanine at position 162 to be an important residue for NHE1 activity and inhibitor sensitivity[40, 41]. Furthermore, mutation of Phenylalanine to Serine renders NHE1 Cariporide-insensitive[41]. Forward primer 5'-

GGCAGCAGGAAGAGGCTGAAGACGTCGGACTG-3' and reverse primer 5'-CAGTCCGACGTCTTCAGCCTCTTCCTGCTGCC-3' were used to generate the F162S mutant via Quikchange mutagenesis kit (Agilent). The mutation was confirmed by DNA sequencing (GeneWiz) using custom sequencing primers 5'-GATCAACAACATCGGCCTCC-3', 5'-GCACCATTCGAAGCTCAGAG-3', and 5'-TTGTCTCCTTCCGTGTTTCA-3'.

HEK293 cell culture and treatment conditions

HEK293 cells were evenly seeded onto 8-chambered coverglass plates (Nunc Lab-Tek) and allowed to reach ~70-80% confluence in DMEM supplemented with 5% fetal calf serum (Atlantic Biologicals). Cells were serum starved (0.1% fetal calf serum) and transfected with either wild-type (WT) or F162S mutant NHE1 plasmid using acidified polyethylenimine (PEI; 3 μg DNA:1 μg PEI). Cells were incubated under standard tissue culture conditions for 48 hours to allow for over-expression of the plasmids.

NHE1 Activity Assay

NHE1 plays a major role in regulating intracellular pH via extruding intracellular H^+ in exchange for sodium. A classic assay of intracellular pH perturbation, ammonium chloride pulse acid loading, was performed as previously described[42] with modifications. HEK293 cells over-expressing WT or F162S NHE1 were loaded with the cell-permeant ratiometric dual emission fluorescent pH indicator SNARF-5F-AM (Molecular Probes)

for 60 minutes at 37°C. Cells were rinsed of residual extracellular SNARF, incubated in a HEPES-buffered Ringer solution (in mM: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4, 10 glucose, 32.2 HEPES), and mounted onto the heated stage of an inverted Olympus Spinning Disk confocal microscope for live-cell imaging. An optical filter set for SNARF fluorescence was obtained from Chroma: excitation 485 ± 25 nm, Q505lp dichroic mirror, emission 585 ± 25 nm and 640 ± 25 nm. The light source was Prior Lumen Pro 220. A 20x objective in wide-field mode was used to monitor cell fluorescence over time.

Upon application of the HEPES buffer, cells were imaged for two minutes at 10 seconds/frame. Ammonium chloride pulse was initiated with a 3-minute incubation of a sodium-free HEPES Ringer solution (in mM: 132.8 NMDG, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 10 glucose, 10 mannitol, 32.2 HEPES) containing 20 mM NH_4Cl, in the presence or absence of ERTU (200 ng/mL) or NHEi (Cariporide, 10 uM). During this time, slight intracellular alkalization occurs as $NH_3 + H^+$ freely enters cells, which can form the weak base NH_4^+ and accumulate. Cells were removed of extracellular NH_4Cl by replacing the solution with sodium-free HEPES Ringer solution (substituting 10 mM NMDG and 10 mM mannitol for the 20 mM NH_4Cl) for 1 minute, imaging at 10 seconds/frame. During this time intracellular NH_4^+ dissociates to NH_3 and H^+. NH_3 freely leaves the cell, and in the absence of extracellular sodium, NHE1 is unable to extrude H^+, resulting in rapid intracellular acidification. Cell media was exchanged for the original HEPES-based Ringer solution, to allow for a recovery period, with 5-second interval image capture, and cells were imaged as intracellular pH returned to homeostasis.

At the end of the recovery period, cells were calibrated to pH 6.5 using a high potassium nigericin/valinomycin-based intracellular pH calibration buffer kit (Molecular Probes). The ratiometric fluorescence at pH 6.5 for each experiment was scaled against a historic *in situ* pH calibration curve acquired at the same imaging settings, again using the calibration buffer kit.

NIS elements software (Nikon) was used to extract ratiometric fluorescence data from regions of interest within the images by dividing background-corrected 640 nm emission by 585 nm emission. The rate of pH_i recovery during the first 75 seconds after addition of Recovery buffer was used to calculate the maximal activity of NHE1.

Statistics

Data shown as mean ± SEM. Statistics were performed using unpaired t-test or ANOVA with bonferroni post-hoc tests, where appropriate, calculated using Graphpad Prism 6. For all analyses, a p-value of < 0.05 was considered to be statistically significant.

RESULTS

Ertugliflozin reduced weight gain and prevented insulin resistance in mice fed a HFHS diet

HFHS-fed mice gained more body weight over time compared to CD-fed mice (1A), which was statistically significant by 1 month on diet and was sustained throughout the duration of the study. As expected, HFHS-fed mice tended to consume more calories compared to CD-fed mice (1B). ERTU-treated mice gained less weight than their diet-matched control groups, despite no appreciable difference in caloric consumption. Mice on HFHS diet had elevated fasting blood glucose (1C), fasting insulin (1D), hemoglobin A1c (1E), and calculated HOMA-IR (1F), compared to CD-fed mice. All of the above were normalized by ERTU treatment, and of note these parameters were not affected by ERTU in CD-fed mice. Together this data indicates that ERTU prevented the development of metabolic syndrome-derived systemic hallmarks.

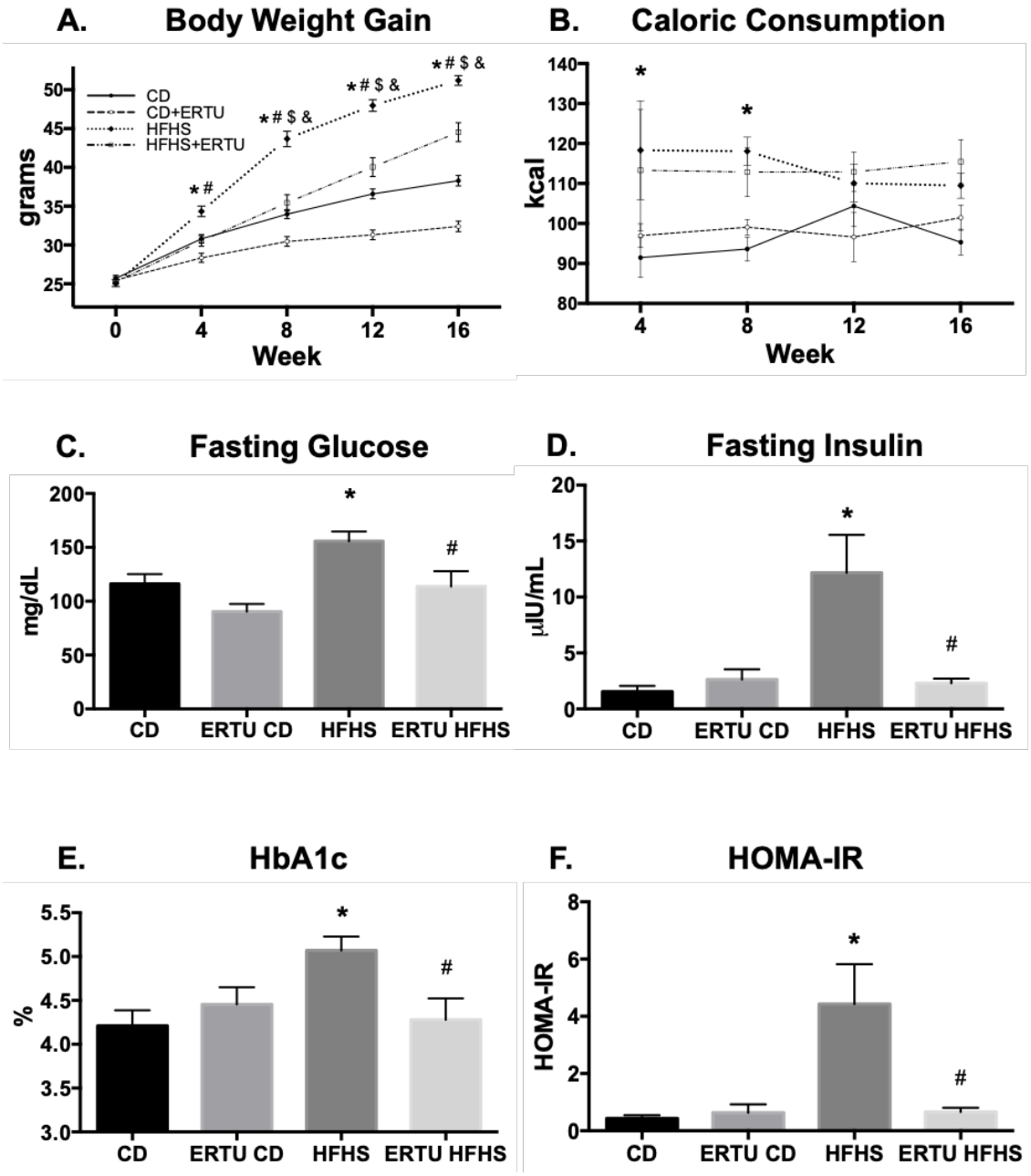

Figure 1: Systemic effects of Ertugliflozin. A) Body weights of mice over 4 months, n=25/group. B) Weekly caloric consumption per mouse over 4 months, n=25/group. C) fasting glucose, D) fasting insulin, E) hemoglobin A1c, F) HOMA-IR calculation after 4 months on diet, n=4-9 per group. * $p<0.05$ CD vs HFHS; # $p<0.05$ HFHS vs ERTU HFHS; $ $p<0.05$ CD vs ERTU CD; & $p<0.05$ ERTU CD vs ERTU HFHS.

Ertugliflozin prevented cardiac hypertrophy and diastolic dysfunction in mice fed a HFHS diet

Metabolic syndrome can progress to metabolic heart disease, characterized in large part by left ventricular hypertrophy and diastolic dysfunction. Consistent with our previous work[33, 43], HFHS-fed mice had increased left ventricular heart weight relative to tibia length (2A), as well as impaired diastolic function as reflected by decreased tissue doppler myocardial relaxation rate (Em; 2B), both of which were prevented by ERTU treatment.

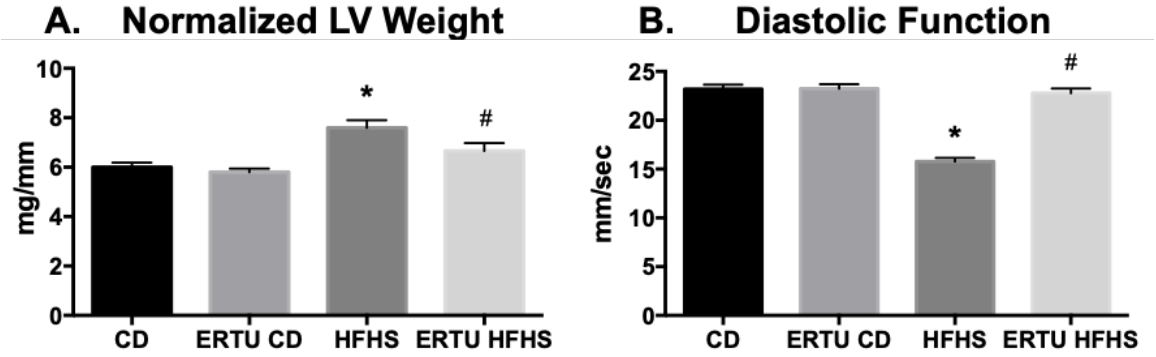

Figure 2: Effects of Ertugliflozin on cardiac structural remodeling in HFHS-fed mice. A) Left ventricular wet weight relative to tibia length, n=12/group. B) Em tissue velocity of relaxation measured by tissue doppler echocardiography, n=25/group.
* $p<0.05$ vs CD; # $p<0.05$ vs HFHS.

Ertugliflozin prevented cardiomyocyte insulin resistance

To remove potential systemic modulators of cardiac phenotype with ERTU treatment, we directly assess effect of ERTU on primary adult rat ventricular myocytes (ARVMs). Using high palmitate treatment to induce insulin resistance, as characterized elsewhere[44], ARVMs then stimulated with insulin exhibited a blunted insulin-induced

phosphorylation of AKT, compared to low palmitate controls (3A-B). ERTU co-treatment of ARVMs under high palmitate conditions restored AKT signaling capacity. The specific NHE1 inhibitor, Cariporide (NHEi), also rescued insulin sensitive AKT signaling in high palmitate co-treated ARVMs. The ability of ERTU to mimic NHEi in preventing insulin resistance in ARVMs suggests that they may share a common mechanism.

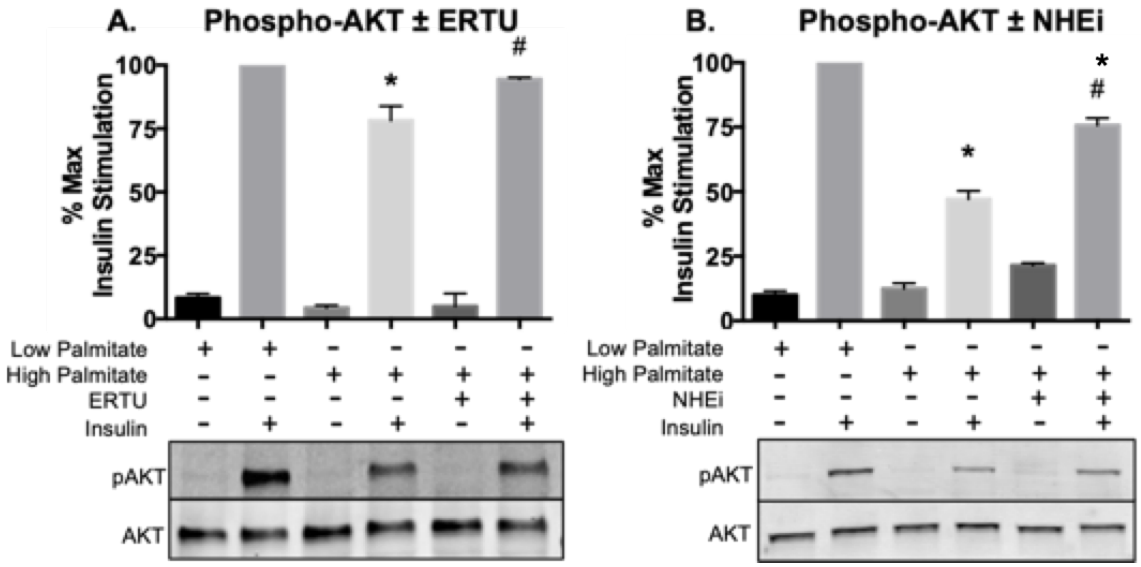

Figure 3: Direct cardiac effects of Ertugliflozin and Cariporide in insulin resistant ARVMs. A) 48 hours low or high palmitate treatment ± ERTU, followed by acute insulin stimulation (100 nM for 15 minutes), phosphorylated and total AKT measured by western blot. B) 48 hours low or high palmitate treatment ± Cariporide, followed by acute insulin stimulation (100 nM for 15 minutes), phosphorylated and total AKT measured by western blot. n=3 primary cell preparations. * $p<0.05$ vs low palmitate + insulin; # $p<0.05$ vs high palmitate + insulin.

Ertugliflozin inhibition of NHE1 activity is prevented by a F162S point mutation

Ammonium pulse acid load assay was used to assess NHE1 activity in HEK293 cells. SNARF-5F-AM ratiometric fluorescence was monitored over the duration of the protocol as a read-out of intracellular pH (4A, example kinetic traces). The ability of NHE1-driven pH recovery was assessed during the first 75 seconds after sodium repletion and expressed as change in pH per minute.

ERTU decreased NHE1 activity by 67% compared to no treatment in cells over-expressing WT NHE1 (4B). As expected, NHE1-specific inhibitor Cariporide (NHEi) blocked NHE1 activity by 95%. In cells over-expressing the F162S point mutation of NHE1, ERTU no longer inhibited NHE1 activity. These data indicate that ERTU acts in a similar fashion to Cariporide in NHE1 blockade, and a site previously known to be important for Cariporide inhibition also appears to be important for ERTU-mediated inhibition.

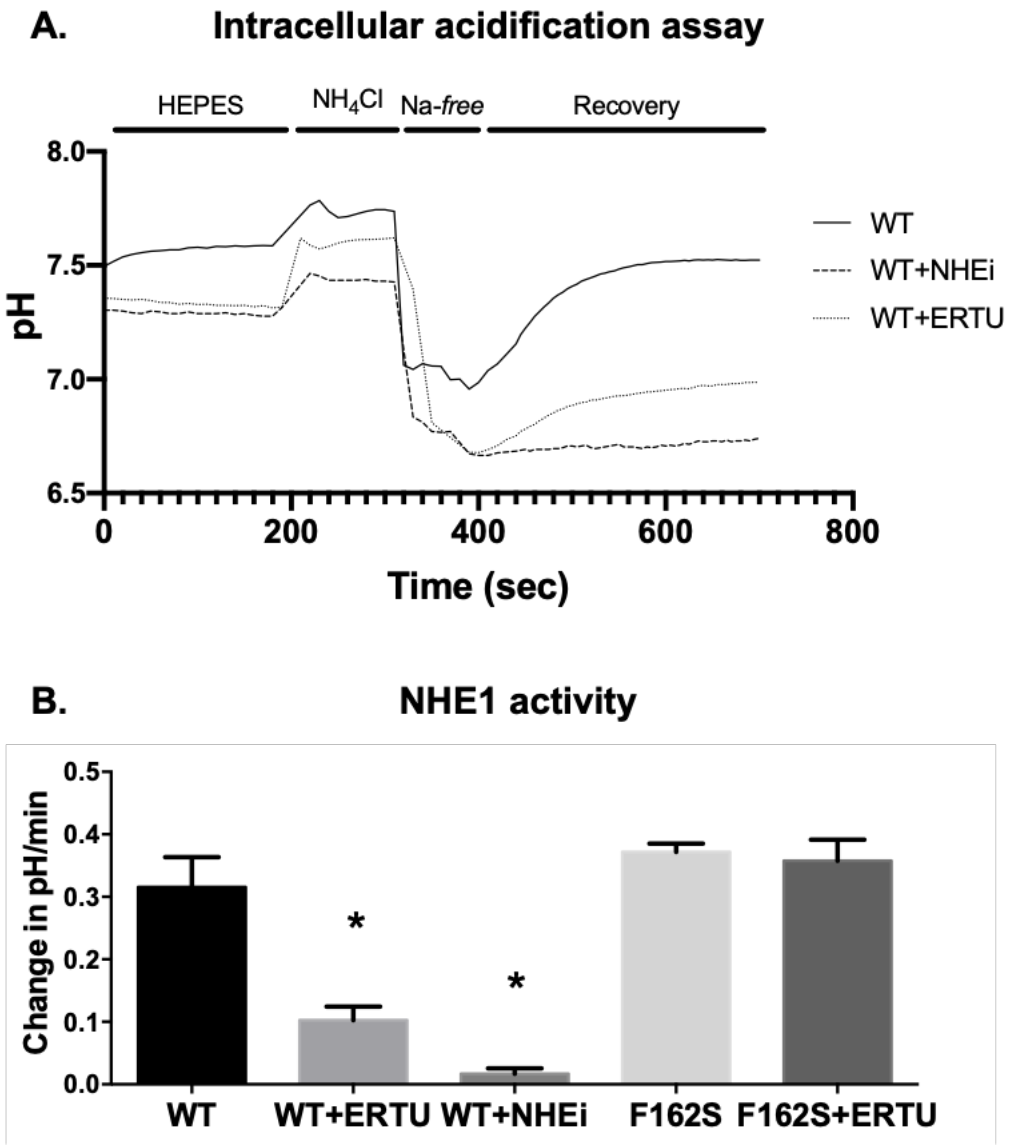

Figure 4: Direct effect of Ertugliflozin on NHE1 activity. A) Design and example of ammonium chloride pulse intracellular acidification assay kinetics over time for WT NHE1 over-expressing cells. B) Calculated NHE1 activity as measured by the intracellular pH recovery over the first 75 seconds of sodium repletion in WT NHE1 and F162S NHE1 ± ERTU. n=4 (n=2 for WT + NHEi), * $p<0.05$ vs WT.

DISCUSSION

This thesis work provides new insight regarding the cardiac effects of Ertugliflozin in a mouse model of metabolic heart disease, and the ability of Ertugliflozin to act directly on the cardiac myocyte *in vitro*. Ertugliflozin prevented the onset of metabolic heart disease in HFHS-fed mice. Notably, our *in vitro* studies show that Ertugliflozin is a direct modulator of insulin sensitivity in the high palmitate-treated cardiomyocyte, and that ERTU-mediated inhibition of NHE1 activity can be prevented by a NHE1 point mutation. Taken together, our studies support the notion that inhibition of NHE1 may contribute to the cardiovascular benefits of SGLT2 inhibitors in metabolic heart disease, and potentially other cardiovascular disease etiologies.

Ertugliflozin prevents the development of metabolic syndrome in HFHS-fed mice

Administration of ERTU with HFHS diet prevented the onset of diabetic hallmarks, consistent with findings of other SGLT2 inhibitors in diabetic models[45–52]. To our knowledge, few studies have approached metabolic syndrome via purely dietary methods[50–52], and ours is the first report using a high fat and high sucrose diet-induced obesity as well as the first using Ertugliflozin specifically. The protection from metabolic syndrome in part can be linked to the reduced body weight gain over time imparted by ERTU, despite no difference in caloric intake. This is likely due to the excretion of glucose in urine. Of note, in ERTU CD animals, although body weights lagged behind their CD counterparts throughout the study duration, hemoglobin A1c, blood glucose, and insulin levels were comparable, supporting the notion that this drug could be safe for

non-diabetics, with low risk of hypoglycemia. Several recent and ongoing clinical studies have been evaluating the effects of SGLT2 inhibitors regardless of diabetic status to treat both heart and kidney disease[18, 20, 53–55].

Ertugliflozin is cardioprotective at least in part via direct action on the myocardium

In addition to ERTU protecting from metabolic syndrome in HFHS-fed mice, ERTU also prevented cardiac hypertrophy and diastolic dysfunction, two major hallmarks of metabolic heart disease seen in diabetics. While in our mouse model it was not feasible to segregate direct versus systemic effects of ERTU on the heart, we subsequently investigated the effect of ERTU on isolated adult rat cardiomyocytes, using a high palmitate treatment to induce an insulin-resistant phenotype *in vitro*. Using this *in vitro* model, we observed that ERTU prevented the high palmitate-induced blunting of insulin signaling, as evidenced by phosphorylation status of AKT. Importantly, this was achieved using a physiologically relevant dose of ERTU[37, 38], suggesting our *in vitro* phenotype is obtainable in *in vivo* settings. Other groups have reported direct effects of SGLT2 inhibitors chronically administered *in vitro* on AMPK[46, 56, 57], GLUT1[58], and Calcium/calmodulin-dependent kinase II[59], but to our knowledge we are the first to report a SGLT2 inhibitor-mediated protective effect on the insulin signaling cascade AKT in adult primary cardiomyocytes.

In addition to ERTU exerting cardioprotective effects on high palmitate-treated cardiomyocytes, Cariporide (NHEi), a specific NHE1 inhibitor, similarly protected cells from insulin insensitivity. It has been previously described that NHE1 becomes more

active in pathophysiological disease states such as diabetes and heart failure[27]. To this end, NHE1 knockout mice exhibit cardiac protection from the effects of high fat diet feeding[60], and NHE1 silencing attenuates cardiac injury and failure[61, 62].

Ertugliflozin directly inhibits NHE1 activity

While previous studies have observed similarities between SGLT2 inhibitors and NHE1 inhibitor action on isolated cardiomyocytes[31, 32] and hearts[32, 63], as well as conducted computational docking studies suggesting a similar binding pocket on NHE1[32], no study to our knowledge has definitively shown that the effect of the SGLT2 inhibitor is due to its binding to the NHE1. Through our HEK293 studies over-expressing WT NHE1, we saw robust inhibition of NHE1 activity with Cariporide and to a lesser extent ERTU. Furthermore, we showed that a single point mutation, F162S, was able to attenuate the ERTU-mediated inhibition, revealing a direct binding mechanism of ERTU and possibly other SGLT2 inhibitors to reduce NHE1 activity. The question remains whether blocking NHE1 activity is responsible for one or more of the beneficial effects on the cardiomyocyte.

How might inhibition of NHE1 prevent insulin resistance? Increased acidification at the extracellular microdomain of the insulin receptor inhibits its ability to fully activate and phosphorylate downstream targets. In skeletal muscle isolated from diabetic OLETF rats, incubation in an acidic medium led to decreases in insulin binding affinity to the receptor, phosphorylation (activation) of the receptor, and decreased phosphorylation of AKT[64]. In primary rat adipocytes, incubation in acidic medium also led to impaired

insulin binding affinity to the insulin receptor and activation, which was dependent on extracellular sodium and inhibitable with NHE1 blocker amiloride[65]. In addition, a high fat milieu can lead to intracellular accumulation and esterification of free fatty acids and intracellular acidification[66], which may in turn activate NHE1 activity resulting in further extracellular acidification. Active NHE1 may contribute to the flux and accumulation of H^+ in the extracellular microdomain and affect insulin sensitivity, and thus we could speculate that blockade of NHE1 protects from localized extracellular acidification, thereby preserving insulin sensitivity at the cellular level.

Oxidative stress is an important mediator of insulin resistance[67–69] and heart failure[70, 71], often perpetuating a vicious cycle. In cardiomyocytes, mitochondria are the major source of reactive oxygen species generation, and thus chief contributors to cellular oxidative stress[72, 73] and implicated in signaling pathways promoting metabolic heart disease hallmarks hypertrophy and diastolic dysfunction[70, 72, 74]. Ion homeostasis is crucial for optimal mitochondrial function and redox functional capacity, with calcium and sodium as integral members co-dependent on each other[75, 76]. Intracellular sodium overload, at least in part due to increased NHE1 activity[77], increases reactive oxygen species emission[67, 76]. In a streptozotocin-induced type 2 diabetic rat model, Empagliflozin treatment attenuated intracellular myocardial sodium levels, NHE1 flux, and reactive oxygen species content[78]. In all, SGLT2 inhibitor treatment may contribute to cardioprotection via restoring ionic balance, limiting reactive oxygen species production, rescuing mitochondria function, and improving insulin sensitivity.

Limitations and future directions

This thesis work provides exciting evidence of direct cardiac action of the SGLT2 inhibitor Ertugliflozin via NHE1 blockade. Our *in vitro* studies could be further bolstered in several ways. NHE1 assays were performed in HEK293 cells to enable transient transfection and high-throughput screening, as primary ARVMs require adenovirus or adeno-associated virus infection in order to over-express proteins of interest. We plan to create adenoviruses for WT and F162S NHE1, overexpress in ARVMs, and examine if NHE1 activity assay findings can be recapitulated, and in addition, determine if beneficial effects of SGLT2 inhibition on cardiomyocyte structure and/or function are mediated via NHE1. Furthermore, introduction of F162S into ARVMs could be used in *in vitro* high palmitate insulin sensitivity studies to further implicate NHE1's role in effective insulin signaling. In the meantime, exploring WT vs F162S NHE1 phenotypes in a cardiomyocyte-like, transfectable cell line, such as AC16 cells, may prove a rapid route to validate the HEK293 studies as well as move forward on revealing mechanistic underpinnings of Ertugliflozin cardioprotection. Ultimately, further investigation is warranted in primary cardiomyocyte studies utilizing NHE1 mutants to conclusively determine whether NHE1 inhibition via Ertugliflozin is responsible for cardioprotection and what mediator(s) are involved—pH, sodium, or a combination of both.

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