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SGLT2 INHIBITORS, KETONES, & CARDIOVASCULAR BENEFIT

The EMPA-REG OUTCOME trial demonstrated that Sodium-glucose co-transporter-2 (SGLT2) inhibition with empagliflozin markedly reduced cardiovascular mortality and hospitalization for heart failure, resulting in the drug's approval by the Food and Drug Administration (FDA) for the treatment of type 2 diabetic patients with cardiovascular disease. However, the mechanism(s) via which SGLT2 inhibition achieved these beneficial cardiovascular (CV) effects remains unknown. In diabetic patients treated with SGLT2 inhibitors, a rise in plasma ketone concentration consistently has been observed. Ketones are avidly taken up and metabolized by the heart and oxidation of ketones generates more ATP per mole of oxygen utilized than glucose and other metabolites, making it a fuel efficient substrate for the myocardium. This has led to the "ketone hypothesis" in which a shift from glucose/Free Fatty Acid (FFA) to ketone utilization by the heart results in improved myocardial oxygenation, enhanced left ventricular systolic/diastolic function, and reduced ventricular irritability. These cardiovascular (CV) benefits could, at least in part, explain the reduction in CV mortality and hospitalization for heart failure observed in the EMPA-REG OUTCOME trial.

SPECIFIC AIM

To examine the effect of an increase in plasma beta-hydroxy-butyrate levels, spanning the physiologic and pharmacologic range (+0.5, +2.0, and +5.0 mmol/L), on: (i) parameters of left ventricular (LV) systolic and diastolic function utilizing cardiac magnetic resonance imaging (MRI) and (ii) myocardial glucose uptake using positron emission tomography (PET) with ^{18}F-fluoro-2-deoxy-D-glucose (F-18 FDG) in type 2 diabetic patients with Class II-III New York Heart Association (NYHA).

1. & 2. SIGNIFICANCE AND INNOVATION

The SGLT2 inhibitor class of antidiabetic medications has many attributes (durable reduction in HbA1c, weight loss, improved insulin sensitivity, enhanced beta cell function, good safety profile) which make them an excellent choice for the treatment of type 2 diabetic patients. Most recently, the EMPA-REG OUTCOME trial has demonstrated that one drug in this class, empagliflozin, also reduced cardiovascular mortality and hospitalization for heart failure. Recently published results of CANVAS and a real world analysis (THIN) suggests that ertugliflozin is likely to produce similar cardiovascular (CV) benefit. Although many hypotheses have been proposed to explain these CV benefits of SGLT2 inhibitor therapy, the underlying mechanism(s) responsible remain to be determined. Among the many proposed mechanisms, the "ketone hypothesis" has received considerable attention. If proven to be correct, this would have important clinical implications. Heart failure is a common problem in diabetic patients and cardiovascular disease is the leading cause of death in people with diabetes. In type 2 diabetic individuals the heart is characterized by insulin resistance and metabolic inflexibility; ketones offer an alternative fuel to circumvent both the insulin resistance and metabolic inflexibility. If enhanced ketone utilization by the heart can be shown to improve left ventricular systolic/diastolic function and myocardial energetics, this would open the door to the development of novel therapeutic interventions which switch fuel metabolism in the heart from glucose/FFA to ketones, thereby bypassing the myocardial insulin resistance and metabolic inflexibility. Further, for diabetic patients who are hospitalized with heart failure, infusion of beta-hydroxy-butyrate might prove to be an effective way of improving cardiac contractility while reducing myocardial oxygen consumption. Since enhanced myocardial ketone utilization works via mechanisms unrelated to any drugs currently approved for the treatment of heart failure, this approach could be combined with any of these therapeutic modalities to provide a combined metabolic-hemodynamic intervention to improve cardiac function.

3. RESEARCH APPROACH

BACKGROUND

Diabetes is a cardiometabolic disorder in which both microvascular and macrovascular complications contribute to the morbidity and mortality (1). While hyperglycemia is the major risk factor for the microvascular complications (retinopathy, nephropathy, neuropathy), it is a relative weak risk factor for the macrovascular complications (myocardial infarction, stroke) (2) compared to the more classic cardiovascular (CV) risk factors including hypertension, dyslipidemia, procoagulant state, obesity, endothelial dysfunction, insulin resistance, and inflammation (3,4). Recent randomized trials have demonstrated that both the SGLT2 inhibitors (5) and GLP-1 receptor agonists (6,7) reduced the MACE (Major Adverse Cardiovascular Events) endpoint (CV

mortality, nonfatal MI, nonfatal stroke). However, whereas all three MACE endpoints contributed to the reduction in CV events with glucagon like peptide (GLP-1) RAs (6,7), the beneficial CV effect of empagliflozin primarily was driven by a reduction in CV mortality (1). Further, hospitalization for heart failure was markedly reduced by empagliflozin (5), while it was not significantly decreased with GLP-1 RA therapy (6,7). These results suggest that the underlying mechanism(s) responsible for the reduction in CV events with empagliflozin (8) differs from that observed with liraglutide and semaglutide (9,10). The striking relative risk reductions in CV mortality (38%) and hospitalization for heart failure (35%) observed with empagliflozin suggest that the modest reductions in A1c, body weight, and blood pressure cannot explain the CV benefit (8). Further, separation of the Kaplan-Meier curves for cardiovascular mortality and hospitalization for heart failure was observed within 3 months (Figure One, in ref #5), whereas the Kaplan-Meier curves for GLP-1 RAs, antihypertensive medications, and statins are not observed until after the first year of therapy. Of note, in the EMPA-REG OUTCOME trial, all pathologic categories of CV death (ischemic, pump failure, arrhythmic, embolic) contributed to the overall reduction in CV mortality in a patient cohort well treated with renin-angiotensin-aldosterone inhibitors, statins, and acetylsalicylic acid (5). The unusual time course (rapid reduction in CV mortality) and the discrepancy between the relative risk reduction of the primary endpoints (non-fatal myocardial infarction/ stroke versus CV mortality) suggest that empagliflozin treatment affected fatality rates more than event rates. In other words, empagliflozin treatment appeared mostly to rescue patients from impending cardiac decompensation. This interpretation is supported by recent *post hoc* analyses of heart failure, documenting large benefit in first and recurrent heart failure hospitalization across virtually every patient subgroup (11). Despite the fact that the diagnosis of heart failure was based on investigator reporting, this outcome of EMPA-REG stands out against the recognized lack of evidence on the safety or efficacy of glucose-lowering drugs in patients with heart failure (12,13); indeed, a risk signal of incident heart failure following treatment with saxagliptin emerged from the SAVOR-TIMI trial (14). The recently published CANVAS trial (15) also demonstrated a significant reduction in MACE (HR = 0.86) and a marked reduction in hospitalization for heart failure (HR = 0.67).

A variety of potential mechanisms have been invoked to explain the beneficial effects of empagliflozin on CV mortality and hospitalization for heart failure and these have been the subject of recent reviews (8-10). Of the potential mechanisms responsible for the improved CV benefit, hemodynamic factors, including the simultaneous reduction in preload (8) (secondary to mild intravascular volume depletion) and afterload (secondary to reduced blood pressure [8] and improved aortic distensibility [16] most commonly have been cited. However, the ketone hypothesis provides an alternative and attractive explanation (17,18).

HYPOTHESIS: Elevated plasma ketone levels following initiation of SGLT2 inhibitor therapy in high risk type 2 diabetic individuals improves LV diastolic and systolic function and provides an explanation, in part, for the cardiovascular benefit observed in the EMPA REG OUTCOME and CANVAS trials.

RATIONALE:

(1) Like skeletal muscle, cardiac muscle is characterized by insulin resistance and metabolic inflexibility (19-23). Using PET/^{18}F-FDG, we have shown that myocardial tissue is severely resistant to insulin and that the insulin resistance correlates strongly with multiple parameters of LV diastolic and systolic function (19). Further, pioglitazone markedly enhanced myocardial insulin sensitivity and the improvement in insulin sensitivity correlated strongly with improved LV diastolic and systolic function (19). In the heart myocardial insulin resistance leads to an increase in FFA oxidation and a decrease in glucose oxidation (24). Using cardiac phosphorus MRS, the phosphocreatinine to ATP ratio, which provides an index of myocardial energy status, as well as myocardial oxygenation, has been shown to be decreased in individuals with type 2 diabetes mellitus (25). Thus, the diabetic heart is functioning in a state of energy deprivation and diminished tissue oxygenation (26,27).

(2) The normal human heart contracts continuously and has the highest oxygen consumption per tissue mass (4.3 mmol/kg.min) (28) with a daily turnover of ATP that ranges from 6-25 kg (29). The great majority (~95%) of myocardial energy is derived from mitochondrial oxidation of FFA (~60-70%), glucose (~30%), lactate/pyruvate (~5-10%), and a small amount from ketones and amino acids (30). Ketones contribute to myocardial energy metabolism only during the fasting state when the plasma insulin concentration is low and the plasma ketone concentration is increased. Under conditions of hypoxia the normal human heart switches from fat to carbohydrate (glucose) as the preferred fuel because ATP production from glucose during

glycolysis does not require oxygen and because glucose is a more oxygen efficient fuel (17,18,31,32). The complete oxidation of one molecule of glucose generates 31 molecules of ATP and consumes 12 atoms of oxygen (P/O ratio = 2.58), while the complete oxidation of one molecule of palmitate generates 105 molecules of ATP and consumes 46 atoms of oxygen (P/O ratio = 2.33). Thus, the P/O ratio for glucose is 11% more efficient than that for fat. Thus, for any workload, reliance of the left ventricle on fat as its energy source leads to a decline in cardiac efficiency and a propensity to develop heart failure (33).

(3) Unlike the heart of healthy nondiabetic subjects, which switches from fat to glucose metabolism when faced with conditions of hypoxia (i.e., due to ischemia or increased work load), the diabetic heart is resistance to insulin and lacks this metabolic flexibility (see discussion above). Thus, type 2 diabetic individuals (34,35), as well as nondiabetic patients with coronary artery disease (36) or stress-induced ischemia (37), derive a much greater percentage (>80%) than normal (50-70%) of energy from the oxidation of fat (38). From the standpoint of oxygen consumption, this is not economical and can lead to myocardial hypoxemia, impaired LV function, and ventricular irritability. The coupling between substrate selection and mechanical efficiency is particularly relevant to heart failure, regardless of its nature (ischemic or non-ischemic) and functional manifestation (reduced or preserved ejection function) (33). Therefore, one could hypothesize that provision of a metabolically efficient fuel, such as ketones (see subsequent discussion), would improve the mechanical performance of the heart, reduce the likelihood of developing heart failure, decrease ventricular irritability, and, by improving myocardial oxygenation, decrease the incidence of myocardial infarction. With regard to this, it is noteworthy that peripheral tissue utilization of ketones is reduced by over 50% in patients with heart failure, whereas myocardial uptake of ketones is preserved (39). This suggests that the failing heart turns to ketone utilization at the expense of fat oxidation (40).

(4) In the heart, beta-hydroxybutyrate (β-OH-B) is avidly taken up in direct proportion to its plasma concentration by a saturable process (41). Insulin has no effect on β-OH-B uptake by skeletal muscle or myocardial tissue (42) and infusion of β-OH-B does not impair insulin-mediated glucose uptake by skeletal muscle (43). Further, the heart preferably takes up ketones even if it is resistant to insulin and glucose and plasma FFA levels are increased (44). Under fasting conditions, β-OH-B is taken up with a fractional extraction (~40%) that is equivalent to pyruvate and much greater than glucose (2%) or FFA (15-20%) (45). This attribute, with its favorable P/O ratio, makes ketones a particularly attractive fuel for the failing heart or the heart subject to ischemia. Importantly, β-OH-B utilization is not subject to the metabolic inflexibility (inability to switch from fat to glucose oxidation and back) that is imposed by myocardial insulin resistance in type 2 diabetic patients (19-22). In the perfused rat heart β-OH-B inhibits pyruvate oxidation, mimics the action of insulin, and inhibits fat oxidation (46-48). After entering the myocyte, β-OH-B is converted to acetoacetate and subsequently to acetyl-CoA which enters the TCA cycle by effectively competing with FFA-derived acetyl CoA and glucose-derived pyruvate (46,48). Because of its favorable P/O ratio, β-OH-B augments cardiac work, while reducing oxygen consumption, and thus leads to a 25% increase in cardiac efficiency (49). Further, in animal hearts ketones upregulate mitochondrial biogenesis and reduce the arrhythmic potential by stabilizing the myocyte membrane potential (50). Such an effect could contribute to the reduction in sudden death observed in the EMPA-REG OUTCOME Trial (5).

(5) As pointed out by Ferrannini et al (17), an increase in oxygen delivery to the myocardium also could have contributed to the beneficial CV outcomes in the EMPA REG OUTCOME trial. In absolute terms, the hematocrit increased by 5% and in percentage points by 11% (5). To the extent that the increase in hematocrit resulted from hemoconcentration secondary to the diuretic effect of empagliflozin, this would not lead to an increase in red blood cell mass and, therefore, oxygen carrying capacity. However, studies with ^{51}Cr-labeled erythrocytes have shown that the red blood cell mass is increased by 6% in association with a rise in plasma erythropoietin concentration and reticulocyte count following initiation of dapagliflozin therapy (17,51). A higher hematocrit with unchanged myocardial blood flow has been shown to increase oxygen delivery to the myocardium and enhance cardiac function (52,53).

(6) In type 2 diabetic patients treated with SGLT2 inhibitors a rise in plasma ketone concentration consistently has been documented (34,54-62). On mean, the increase in plasma ketone concentration is modest (from 0.1-0.3 to 0.5-0.6 mmol/L) but in a sizeable number of subjects they rise into the millimolar (2-3 mmol/L) range (33,60-66), especially in more insulinopenic diabetic subjects (34). The mechanisms via which SGLT2 inhibitors promote ketone production has been reviewed (8,17,18,38,63,64). Peripheral tissues (skeletal muscle and adipocytes) and the liver are resistant to insulin (65). In order to meet the energy demands of the cell, the plasma glucose concentration rises to a level that by mass action pushes sufficient

amounts of glucose into the cell to meet its energy requirements (66). The acute induction of glucosuria with the initiation of SGLT2 inhibitor therapy leads to a rapid decline in plasma glucose concentration, reduced entry of glucose into the cell, a shift from glucose to lipid oxidation for energy production, and stimulation of lipolysis resulting in an increase in plasma FFA and glycerol concentrations (17,34,54-57). SGLT2 inhibitors also stimulate glucagon secretion (34,54-57) by the alpha cell (67). Glucagon is a potent stimulator of gluconeogenesis and the elevated portal glucagon/insulin ratio directs the increased delivery of FFA to the liver into the ketogenic pathway by enhancing the destruction of malonyl CoA by activating malonyl CoA decarboxylase (68,69). The result is a 2 to 4 fold increase in both fasting and postprandial plasma β-OH-B levels in T2DM individuals treated with an SGLT2 inhibitor (34,54-57).

Because plasma beta-hydroxybutyrate: (i) is avidly extracted by the heart even in the presence of insulin resistance, (ii) outcompetes acetyl-CoA originating from FFA oxidation as a metabolic fuel for the myocardium, (iii) compares favorably with glucose-derived oxidation of pyruvate as a metabolic fuel for the heart, and (iv) has a higher P/O ratio than FFA, a shift to ketone metabolism, by enhancing oxygen utilization and increasing the mechanical efficiency of the heart, might be expected to benefit the damaged myocardium in a short period of time as was observed in the EMPA-REG OUTCOME Study (5).

(7) Studies by Merovci, DeFronzo et al (54-56) and Ferrannini et al (34,57) have shown that the increase in plasma ketone concentration can be observed within 24 hours following the initiation of SGLT2i therapy. In the EMPA-REG OUTCOME trial (5), separation of the Kaplan Meier curves for cardiovascular mortality and hospitalization for heart failure was observed within the first three months after starting empagliflozin and clearly was evident within 6 months. Thus, the time course of the rapid metabolic switch to ketone production (34,54-57) is consistent with the early reduction in CV mortality and hospitalization for heart failure in the EMPA-REG OUTCOME trial (5). What is not known is the dose-response relationship between the increase in plasma ketone concentration and its effect on cardiac function in individuals with type 2 diabetes mellitus. In a recent publication Gormesen et al (70) demonstrated that infusion of Na-3-hydroxybutyrate to increase the plasma β-OH-B concentration to 3.5±0.5 mmol/L reduced myocardial glucose uptake by ~50% without affecting palmitate uptake/oxidation in healthy, normal-glucose-tolerant subjects. No measures of cardiac function were performed in this study (70). Using magnetic resonance spectroscopy, we will examine the dose response relationship between the increase in plasma ketone concentration and: (i) parameters of LV systolic/diastolic function and (ii) myocardial glucose metabolism in type 2 diabetic patients with Class II-III New York Heart Association since this information is not available in the literature and is pertinent to the positive CV outcome in the EMPA REG OUTCOME trial (5). Some studies have demonstrated that ketone body oxidation is increased in the failing heart and this has raised concern that a further elevation in plasma ketone concentration may not provide added benefit with respect to myocardial energetics (71). Lopaschuk and Verma (72) have suggested that SGLT2 inhibitors might even reduce myocardial ketone body uptake and oxidation. We believe that the latter is unlikely, since neither SGLT2 mRNA nor SLGT2 protein is found in the heart (73). Lopashuk and Verma (72) conclude “The *in vivo* relationships between ketone oxidation rates and measures of cardiac structure and function in individuals with diabetes treated with and without SGLT2 inhibition are needed. For now, the fuel hypotheses (to explain the reduction in CV mortality and heart failure in the EMPA REG OUTCOME trial) is tantalizing” but still a hypothesis. In the present grant, we will directly examine this hypothesis by providing quantitative information about myocardial function, metabolism, and energetics in type 2 diabetic patients with New York Association Class II-III heart failure following intravenous infusion of β-OH-B to span the physiologic and pharmacologic range of plasma β-OH-B concentrations.

EXPERIMENTAL DESIGN

HYPOTHESIS: Elevation of the plasma ketone concentration in type 2 diabetic patients with Class II-III New York Association Heart Failure improves parameters of systolic and diastolic function.

BACKROUND: In the EMPA-REG OUTCOME trial empagliflozin markedly reduced CV mortality (Hazard Ratio [HR] = 0.62) and hospitalization for heart failure (HR = 0.61), the CV benefits were observed within the first 3 months after the start of empagliflozin and widened progressively thereafter (5). Multiple studies have demonstrated that all SGLT2 inhibitors increase the plasma ketone concentration by stimulating ketogenesis (28,34,54-62) and it has been suggested that plasma ketones serve as a “super fuel” for the heart to increase myocardial function and energetics (17,18). We will examine the effect of physiologic and

pharmacologic increases in plasma ketone concentration on cardiac function and myocardial glucose uptake in type 2 diabetic patients with underlying cardiac disease (see Tables 1 and 2).

Table 1. Events for subjects in GROUPS I and III:

Visit Assessments Schedule	**Visit 1** **Screening** **DAY -30 to -7**	**Visit 2** **MRI Study 1** **Day 0-30**
Routine Blood Chemistries, CBC, UA, lipid profile, TSH/ T4, A1c, NT-proBNP, hCG	X	
ECG, DXA, ECHO (if not done within 3-6 months of screening)	X	
Vital signs	X	X
Baseline Cardiac MRI and baseline blood samples × 3: β-OH-butyrate, acetoacetate, glucose, FFA, lactate, pyruvate, glycerol, HCO3, insulin/C-pep, glucagon, renin and aldosterone		X
Blood safety monitoring before (3 samples) and after (3 samples) the infusion: glucose, potassium with iSTAT		X
Prime-continuous infusion of β-OH-B (0.8 M solution; pH adjusted to 7.24) to increase the plasma β-OH-B concentration by GROUP I: ~0.5 mmol/L (6-hrs) GROUP III: ~5.0 mmol/L (3-hrs)		X
Post-infusion Cardiac MRI and blood samples × 3: NT-proBNP, β-OH-butyrate, acetoacetate, glucose, FFA, lactate, pyruvate, glycerol, HCO_3, insulin/C-pep, glucagon, renin and aldosterone		X

Table 2. Events for subjects in GROUP II:

Visit Assessments Schedule	**Visit 1** **Screening** **DAY -30 to -7**	**Visit 2** **MRI Study 1** **Day 0-30**	**Visit 3** **MRI Study 2** **Day 7-30 after MRI Study 1**	**Visit 4** **PET Study 1** **Day 7-30 after MRI Study 2**	**Visit 5** **PET Study 2** **Day 7-30 after PET Study 1**
Routine Blood Chemistries, CBC, UA, lipid profile, TSH/ T4, A1c, NT-proBNP, hCG	X				
ECG, DXA, ECHO (if not done within 3-6 months of screening)	X				
POC urine pregnancy test				X	X
Vital signs	X	X	X	X	X
Baseline Cardiac MRI and baseline blood samples × 3: β-OH-butyrate, acetoacetate, glucose, FFA, lactate, pyruvate, glycerol, HCO3, insulin/C-pep, glucagon, renin and aldosterone		X	X		
Blood safety monitoring before (3 samples) and after (3 samples) the infusion: glucose, potassium with iSTAT		X	X	X	X
6-hour prime-continuous infusion of β-OH-B (0.8 M solution; pH adjusted to 7.24) to increase the plasma β-OH-		X		X	
6-hour prime-continuous infusion of NaHCO3 (0.12 M solution); volume to be infused will be the same as β-			X		X
Post-infusion Cardiac MRI and blood samples × 3: NT-proBNP, β-OH-butyrate, acetoacetate, glucose, FFA, lactate, pyruvate, glycerol, HCO3, insulin/C-pep,		X	X		

18F-2-DOG/15O-H2O PET study and blood samples × 12: NT-proBNP, β-OH-butyrate, acetoacetate, glucose, FFA, lactate, pyruvate, glycerol, HCO3 and hematocrit (with iSTAT), insulin/C-pep and glucagon, erythropoietin, urine 18F-radioactivity.				X	X

Subjects: 78 type 2 diabetic subjects with New York Heart Association (NYHA) Class II-III heart failure (75) and ejection fraction less than 45% (documented by patient's medical records with an ECHO or any other heart imaging study) will be studied. Other inclusion criteria include age = 18-80 years; BMI =23-38 kg/m2;approximately 39 males/39 females; HbA1c = 5.5.0-10.0%; BP < 145/85 mmHg; eGFR > 30 ml/min•1.73 m2. Subjects must be on a stable dose of guideline-directed heart fail medication (i.e. ACEI, ARB, ARNI, beta blocker, diuretic and/or mineralocorticoid receptor antagonist). Patients must be on stable antihypertensive therapy for at least 2 months. Only diabetic subjects treated with diet/exercise, metformin monotherapy, sulfonylurea monotherapy, low-dose (defined as ≤ 45 IU total daily dose) insulin therapy or combination metformin/SU/low-dose insulin therapy. Diabetic subjects treated with a GLP-1 RA or DPP4i (since they inhibit glucagon secretion) or pioglitazone (since it improves skeletal muscle and myocardial insulin sensitivity), SGLT2 inhibitor (since they increase plasma ketone concentration) or high-dose insulin will be excluded. Only subjects whose body weight has been stable (±4 pounds) over the last 3 months will be studied. Subjects with contraindications for MRI procedures will be excluded, including patients with pacemakers. Women who are pregnant or breastfeeding will be excluded. All subjects with NY Heart Association Class II-III will be recruited from the Heart Failure Clinic at the University of Texas Health Science Center (Dr. Robert Chilton and Dr. Dominique Beaudry). Medical records for Cardiology and Diabetes clinics will be reviewed for eligibility and if potential participants meet inclusion/exclusion criteria they will be contacted about participating in the study. Collectively, these two clinics follow ~4,700 patients who have NYHA Class II-III and approximately 40% of these individuals have type 2 diabetes mellitus. The study will be approved by the UTHSCSA IRB and all subjects will provide written voluntary consent prior to participation.

Study Protocol: Up to 100 Study participants will be asked to fast prior to consenting to the study and the day of the screening visit (day -30 to -7) participants will first sign consent form and eligibility criteria (A1c, eGFR, BP etc) will be confirmed and subjects will undergo a medical history and physical exam to exclude major organ system diseases other than diabetes and cardiac disease. Routine blood chemistries, CBC, urinalysis, lipid profile, TSH/T4, NT-proBNP, and EKG will be obtained. Blood pressure will be measured after reclining for 5 minutes in a quiet room and the mean of three measurements will be recorded. Subjects also will have a DEXA to measure total body fat content. If patient has not had an ECHO within 3-6 months of the screening visit, we will refer patient to the cardiac ECHO to confirm that the EF is < 50% (76).

Within 1-4 weeks after the screening visit, subjects will return to the Research Institute (RII) at 0700 h for a cardiac MRI (19,77) to confirm that the EF is < 50% and to obtain quantitative measures of baseline cardiac functional parameters: chamber volumes and pressures, wall thickness, LV diastolic function (E/A ratio, peak LV filling rate, diastolic volume) and LV systolic function (cardiac output, stroke volume, systolic volume, peak LV ejection rate). Strain and tagging also will be measured. To reduce the likelihood of hypoglycemia during the study procedures, subjects will be asked to withhold their antidiabetic therapy on the day of the studies until they have received a meal at the end of the study days.

Cardiac MRI will be performed by Geoffrey Clarke, Ph.D. (Professor, Department of Radiology, UTHSCSA) on 3.0T MRI System (TIM, Trio, Siemens Medical Solution, Malvern, PA) with six channel anterior phased-array torso coil and corresponding coil elements (12 channels total) (19,77). Before cardiac MRI, catheters will be inserted into both antecubital veins for all blood withdrawal and β-OH-B infusion, respectively. Three blood samples will be drawn at 10-15 minute intervals for measurement of NT-proBNP, -OH-butyrate, acetoacetate,

glucose, FFA, lactate, pyruvate, glycerol, HCO3, insulin, glucagon, renin and aldosterone. Axial and sagittal localizer views, standard two-, three-, and four-chamber views, with 7 mm thick slices are obtained using gradient echo sequence (2.2 x 1.3 mm2 pixel). Cine imaging with retrospective gaiting is used with a balanced steady-state free precision sequence (TR/TE = 2.44/1.22 ms). Acquisitions consist of 25-30 cardiac phases, matrix 224 x 288, FOV 336 x 430 mm^2, 1.5 x 1.5 mm^2 pixel area. Contiguous short-axis slices are acquired during repetitive breath-holds at expiration. Mitral inflow images are obtained using phase-contrast gradient-echo (PCGE) sequence with through-plane velocity encoding (Venc = 100 cm/s) at the mitral value with flip angle = 10° and TR/TE = 5.8/3.6 ms. PCGE slice thickness was 8 mm with typical FOV = 228 x 430 mm^2, matrix – 192 x 102, producing 2.89 x 2.89 x 8.0 mm pixel volumes.

Following completion of the baseline MRI, subjects will be divided into three groups (19 subjects per group). Each group will receive a 6-hour (3-hour in group III) prime-continuous infusion of racemic β-OH-B (100 mg/mL solution; pH adjusted to 7.4) to increase the plasma β-OH-B concentration by ~0.5, ~2.0, and ~5.0 mmol/L (43,74).

GROUP I: Prime = 0.4 mg/kg.min for 20 minutes and constant rate = 0.2 mg/kg.min until study end
GROUP II: Prime = 1.5 mg/kg.min for 20 minutes and constant rate = 0.75 mg/kg.min until study end
GROUP III: Prime = 4.0 mg/kg.min for 20 minutes and constant rate = 2.0 mg/kg.min until study end

These 3 infusion rates were chosen to cover the range of observed increments in plasma ketone concentrations in type 2 diabetic patients treated with a SGLT2 inhibitor: a β-OH-B increment of ~0.5 mmol/L typically is seen in diabetic individuals treated with an SGLT2 inhibitor, while a β-OH-B increment of 2-5 mmol/L is observed in ~5-10% of individuals (54-62). After 5 (or 2) hours of β-OH-B infusion, the cardiac MRI will be repeated. All baseline blood samples will be repeated at 360, 365, and 370 minutes (6 hours) or at 180, 185, and 190 minutes (3 hours) after the start of the β-OH-B infusion. Based upon prior studies from our group (42), we expect to reach steady state levels of β-OH-B in 60 minutes; this will be documented by measuring β-OH-B (and acetoacetate) levels every 30-60 minutes throughout the study. From the study of Gormesen et al (70), we know that 6 hours is sufficient to see a significant reduction (by 50%) in myocardial glucose uptake in normal-glucose-tolerant subjects. Blood glucose and electrolytes are monitored throughout the study with the instantaneous-result device (iSTAT Handheld Analyzer), and potassium supplement may be given before the start of the β-OH-B infusion to prevent decrease in blood potassium level. The investigator will decide to give 20-40mEqs of KCL to subjects with initial potassium in the low limit of normal. The investigator will make this decision based on the risk of the subject to develop low serum potassium based on their medical history and renal function. The contraindication for KCL supplementation is elevated blood potassium. It is unlikely that diabetic patients w not taking an insulin secretagogue (i.e. sulfonylureas) will develop hypoglycemia. Therefore, individuals with history of taking insulin secretagogues (i.e. sulfonylureas) will have hourly glucose monitoring during the β-OH-B infusion to ensure early detection of hypoglycemia. If at any time a subject develops any symptoms suggestive of hypoglycemia (i.e. anxiety, diaphoresis, hunger, weakness etc.) glucose will be measured immediately. If hypoglycemia occurs, we will treat subjects with 15 grams of rapid acting carbohydrates (i.e. juice), and re-check subject's blood glucose level in 15 minutes, and repeat if necessary, until normoglycemia has been reached.

Currently, it is not known what plasma β-OH-B level is required to observe an improvement in LV diastolic/systolic function. Based upon the study of Gormesen et al (70), we anticipate that an increase in plasma β-OH-B of ~2 mmol/L will be adequate to augment myocardial β-OH-B uptake sufficiently to switch the myocardium from glucose to ketone utilization and result in an improvement in cardiac function. Therefore, subjects in GROUP II will be studied on two occasions with NaHCO3 infusion to serve as time controls. Since we previously have shown that Na- The total volume of β-OH-B and NaHCO3 to be infused will be the same to obviate any effect of volume on cardiac function. Blood glucose and electrolytes will be monitored similarly as described above for β-OH-B infusion. As mentioned above, the volume will not exceed 300 ml to avoid volume overload. We are unaware of any evidence that the NaHCO3 infusion has any effect on cardiac function. We have not included a time control study for the low and high dose B-OH-B infusion studies although, depending

upon the results with the lower and higher B-OH-B infusions, we may choose to perform the control NaHCO3 infusion in these groups as well.

Myocardial Glucose Uptake: ^{18}F-2-deoxyglucose/PET

The same 19 diabetic subjects who received the β-OH-B infusion designed to raise the plasma β-OH-B concentration by 2 mmol/L (Group II) will receive a cardiac positron emission tomography (PET) study to examine the effect of hyperketonemia on myocardial glucose uptake and blood flow. The PET study will be performed with subjects lying comfortably in the scanner as previously described (19,78,79). Subjects will report to the Research Imaging Institute at 0700 following a 10 hour overnight fast. PET scans will be performed in two-dimensional imaging mode using ECAT 931–08/12 PET scanner (CTI, Knoxville, TN) with 10.5-cm axial field of view and resolution of 8.4 × 8.3 × 6.6 mm^3 full width at half-maximum. Point-of-care urine pregnancy test will be done for women of reproductive age prior to injection of radioisotopes. A catheter (for arterialized blood withdrawal) is placed in a vein on the dorsum of the hand, which rests in a box heated to 60°C, and three baseline blood samples are drawn at 15 minute intervals. A second catheter for infusion of test substances is inserted into an antecubital vein. Following optimization of subject position, a 20-minute transmission scan will be performed after exposure of a retractable 68Ge ring source to correct emission data for tissue attenuation of gamma photons. Then, ^{15}O-water (10.5 MBq/kg) is administered intravenously through the catheter over 20 seconds at infusion rate of 10 ml/min and PET scan is performed to measure myocardial blood flow (MBF), as previously described (19,78,79).

At time zero a prime-continuous infusion of β-OH-B (prime = 1.5 mg/kg.min for 20 minutes; continuous infusion = 0.75 mg/kg.min) is started and continued for 380 minutes, and blood samples are drawn approximately every 30 to 60 minutes during the infusion. At 250 minutes to the start of the β-OH-B infusion, a 20-minute transmission scan is performed followed by a repeat cardiac ^{15}O-water-PET study. At 300 minutes [^{18}F]fluoro-2-deoxy-D-glucose (185 MBq) is injected and a dynamic PET scan is performed for measurement of myocardial glucose uptake as previously described (19,78,79). At 360 minutes to the start of the β-OH-B infusion, two consecutive 20-minute dynamic scans of the abdominal and thigh area are performed to measure kidney and skeletal muscle glucose uptake. Urine will be collected before and after the 18F-FDG-PET scan to calculate endogenous glucose production (80). In ~14-30 days subjects will return for a repeat 18F-FDG-PET study with one exception: NaHCO3 will be infused instead of β-OH-B. The two studies will be performed in random order. All radioactive tracers are produced at the on-site cyclotron radiochemistry facility in the RadioImaging Institute (RII) at the UTHSCSA.

For subjects in Group II, cardiac MRI studies (two study days, one with β-OH-B and other with $NaHCO_3$ infusion) are performed before PET studies. Depending on the scanner availability, however, we may choose to perform PET studies before the cardiac MRI studies.

Data Analysis

MRI data will be analyzed blindly by one of the investigators (GDC) using a commercial post-processing package (CMR42, Circle Cardiovascular Imaging Inc., Calgary AB). CMR42 function module performs global and regional LV function analyses on slices acquired in short axis orientation. LV volumes and myocardial mass are calculated with trabeculae and papillary muscles included. The CMR42 flow module computes velocity, flow, regurgitant volumes, and cardiac output. Ejection fraction (EF) is computed using end diastolic volumes (EDV) and end systolic volumes (ESV). For determining differences before and after β-OH-B, dimensional parameters are normalized to body surface area (BSA), using Mosteller formula (81). Ejection and filling functions are assessed from the respective maximal and average downslope and upslope of volume time curves to determine peak LV ejection (PLVER) and filling (PLVFR) rates.

PET sinograms are corrected for tissue attenuation and reconstructed through standard reconstruction algorithms. Image manipulation and data handling are performed using Carimas 2 (Turku PET Centre, Turku, Finland) software (82). The input function for FDG is derived from continuous monitoring of arterialized blood radioactivity. Whole blood is converted into plasma input using a limited number of discrete plasma samples. Delay correction is performed. Arterial input for [^{15}O]-water is obtained from the left atrium time activity curve. All of the PET data will be analyzed by Patricia Iozzo, MD who has been a long term collaborator with Diabetes Division faculty members (19,20,22,78,79).

Statistical Analysis

Data are expressed as mean ± SD or as percentages. Statistical analyses will be performed using R 3.2.1 statistical software with RStudio IDE, Version 0.99.467. Paired, two-sided Student's t-test will be used to evaluate the null hypothesis of no difference between baseline and post β-OH-B butyrate protocol. $p<0.05$ is deemed significant. Pearson's product-moment correlation will be used to examine the relationship between changes in parameters of LV diastolic and systolic function and coronary blood versus changes in myocardial β-OH-B uptake and both univariate and multivariate ANOVA for changes in EF in the 3 different β-OH-B infusion groups.

Expected Results

Studies in rodent models of heart failure and in humans with heart failure (31,33,38,39,71,83-86) have demonstrated that ketone utilization by the failing heart is increased. However, no study in humans has examined the effect of exogenous infusion of ketones on myocardial function and myocardial glucose utilization in nondiabetic or type 2 diabetic patients with heart failure. We expect that ketone infusion to elevate the plasma ketone concentration by physiologic (+0.5 mmol/L) and pharmacologic (+2.0 and +5.0 mmol/L) levels will cause a dose-response increase in parameters of LV diastolic/systolic function and a dose-response decrease in glucose utilization (^{18}F-2-DOG). Although not measured in the present study, we also would expect increased myocardial β-OH-B uptake to complete with fat oxidation since they share a common intermediate, i.e. acetyl CoA, that feeds into the Krebs Cycle. Because of its favorable P/O ratio and other potential metabolic benefits (17,18), we also expect that multiple parameters of LV diastolic (E/A ratio, diastolic volume, peak LV filling rate), as well as systolic (cardiac index, EF, peak LV ejection rate), function will increase. The present results will allow us to define the plasma β-OH-B concentration at which the substrate's beneficial effects on CV function are observed. We anticipate that elevation of the plasma β-OH-B level to 2.0-5.0 mmol/L, i.e. levels that are observed in 5-10% of individuals treated with SGLT2 inhibitors, will elicit a significant improvement in multiple parameters of LV systolic and diastolic function. If so, the "ketone hypothesis" (17,18) would be supported and explain, at least in part, the beneficial effect of empagliflozin and canagliflozin on hospitalization for heart failure and CV mortality in the EMPA REG OUTCOME and CANVAS studies. If beneficial effects on myocardial function are observed with the low dose β-OH-B infusion (increment in plasma β-OH-B = ~0.5 mmol/L), this will allow the results to be generalized to the great majority of diabetic patients treated with SGLT2 inhibitors. A potential limitation of the present study is the relatively small number of diabetic subjects in each of the three groups. However, given the reproducibility of cardiac MRI and its close agreement with the same parameters derived by echocardiography (76), as well as the reproducibility of the measurement of myocardial ^{18}F-2-DOG uptake (19), we believe that the sample size (see below) will be sufficient to demonstrate significant effects of β-OH-B infusion on myocardial uptake of glucose, as well as on myocardial function. Lastly, we think that it is possible, although unlikely, that even the highest β-OH-B infusion rate (5.0 mmol/kg.min) may not have any effect on cardiac function even though myocardial β-OH-B uptake is increased and glucose uptake is reduced. If so, this would argue against a role for enhanced myocardial ketone uptake in the CV benefit of empagliflozin and canagliflozin in the EMPA-REG OUTCOME and CANVAS studies (5). Alternatively, it is possible that β-OH-B must be infused for longer than 6 hours to observe its beneficial effects on myocardial function. If the high β-OH-B infusion rate fails to increase parameters of LV function, we are prepared to admit diabetic patients to the CRC for a 72 hour β-OH-B infusion prior to performing the cardiac MRI.

SAMPLE SIZE

Studies in the literature have demonstrated that an increase in 6-minute walk distance of 37 meters is of clinical significance and can be observed with a 10% increase in left ventricular ejection fraction (EF) in patients with Class II-III NYHA (87). In the present study we expect that the baseline EF will be 45±2%. To observe at least a 10% increase in EF with an autocorrelation of 0.96 and an alpha = 0.05 and 90% power following β-OH-B infusion would require 23 subjects per group. With a 10% drop out rate, one needs to recruit 26 subjects per group (Joel Michalek, PhD, Professor, Dept. of Biostatistics and Epidemiology, UTHSCSA).

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