THE ROLE OF HEAT SHOCK PROTEIN 72 IN ENDOTHELIAL INSULIN RESISTANCE IN TYPE 2 DIABETES:

USING PASSIVE HEAT THERAPY TO IMPROVE ENDOTHELIAL INSULIN RESPONSIVENESS

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by
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THE ROLE OF HEAT SHOCK PROTEIN 72 IN VASCULAR INSULIN RESISTANCE IN TYPE 2 DIABETES:

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I dedicate this dissertation to my wife, Heather.

There are no words that fully express my gratitude for her love and support. I simply couldn't have done this without her.

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ABBREVIATIONS

ANOVA – Analysis of Variance

AP-1 – Activator Protein 1

BMI – Body Mass Index

BSA – Bovine Serum Albumin

CVD – Cardiovascular Disease

DBP – Diastolic Blood Pressure

DEXA – Dual-Energy X-ray Absorptiometry

ELISA – Enzyme-Linked Immunoassay

eNOS – Endothelial Nitric Oxide Synthase

ER – Endoplasmic Reticulum

ERK – Extracellular Regulated Kinase

ET-1 – Endothelin-1

FBS – Fetal Bovine Serum

FMD – Flow-mediated Dilation

FFAs – Free Fatty Acids

GSK-3 – Glycogen Synthase Kinase 3

HA-VSMC – Human Aortic Vascular Smooth Muscle Cell

HbA1c – Hemoglobin A1c

HFD – High-Fat-Diet

HSF-1 – Heat Shock Factor-1

HSP – Heat Shock Protein

hSMMECs – Human Skeletal Muscle Microvascular Endothelial Cells

HOMA-IR – Homeostatic model assessment for insulin resistance

HUVECs – Human Umbilical Vascular Endothelial Cells

iAUC – Incremental Area-Under-the-Curve

IL-1 β – interleukin 1 Beta

IL-6 – interleukin 6

IR – Insulin Resistance

IRS-1 – Insulin Receptor Substrate-1

IUD – Intrauterine Device

JNK – c-Jun amino-terminal kinase

L-NMMA – N^G-monomethyl-l-arginine

MAP – Mean Arterial Pressure

MAPK – Mitogen-Activated Protein Kinase

NFκB – Necrosis Factor Kappa B

NO – Nitric Oxide

NOS – Nitric Oxide Synthase

PAD – Peripheral Arterial Disease

PBS – Phosphate Buffered Saline

PCOS – Polycystic Ovary Syndrome

PI3K - Phosphoinositide 3-Kinase

ROS – Reactive Oxygen Species

RPE – Rating of Perceived Exertion

SBP – Systolic Blood Pressure

siRNA – Small Interfering Ribonucleic Acid

T2D – Type 2 Diabetes

TNFα – Tumor Necrosis Factor Alpha

TMT – Tympanic Membrane Temperature

USG – Urine Specific Gravity

VAT – Visceral Adipose Tissue

VCAM-1 – Vascular Cellular Adhesion Molecule-1

vWF - von Willebrand Factor

ABSTRACT

Impaired endothelial insulin signaling and consequent blunting of insulin-induced vasodilation is a feature of type 2 diabetes (T2D) and contributes to vascular disease and glycemic dysregulation. However, the molecular mechanisms underlying endothelial insulin resistance remain poorly known. Herein, the hypothesis that endothelial insulin resistance in T2D is attributed to reduced expression of HSP72 was tested. HSP72 is a cytoprotective chaperone protein that can be upregulated with heating and is reported to promote insulin sensitivity in metabolically active tissues, in part via inhibition of JNK activity. Accordingly, it was further hypothesized that, in T2D individuals, seven days of passive heat treatment via hot water immersion to waist-level (one hour/day) would improve leg blood flow responses to an oral glucose load (*i.e.*, endogenous insulin stimulation) via induction of endothelial HSP72. Contrary to the hypotheses, it was found that: 1) endothelial insulin resistance in T2D mice and humans was not associated with reduced HSP72 in aortas and endothelial cells, respectively; 2) after passive heat treatment, improved leg blood flow responses to an oral glucose load did not parallel with increased endothelial HSP72; 3) downregulation of HSP72 (via small-interfering RNA) or upregulation of HSP72 (via heating) in cultured endothelial cells did not impair or enhance insulin signaling (*i.e.*, activation of Akt), respectively, nor was JNK activity altered. Collectively, these findings do not support the hypothesis that reduced HSP72 is a key driver of endothelial insulin resistance in T2D but provide novel evidence

that lower-body heating may be an effective strategy for improving postprandial blood flow in subjects with T2D.

CHAPTER ONE – BACKGROUND & AIMS

The prevalence and incidence of type 2 diabetes (T2D) are growing in the United States and worldwide (1, 2). The number of US adults diagnosed with diabetes is expected to nearly triple by 2060, equating to over one in six adults (3). The increased rates of T2D are mirrored by the increased prevalence of obesity (4, 5) and physical inactivity (6, 7), known prime drivers of T2D (8). Notably, individuals with T2D are at increased risk of developing and subsequently dying from cardiovascular disease (CVD) (9, 10). Individuals with T2D display vascular dysfunctions that manifest as hypertension, arterial stiffening, endothelial dysfunction, and impaired insulin-stimulated increases in skeletal muscle blood flow and perfusion (11). Much of these manifestations stem from complex endothelial cell mechanisms (12-14). Notably, these vascular dysfunctions contribute to the increased risk of CVD associated with T2D (15), yet the mechanistic and molecular causes are not yet fully understood.

Endothelial cell dysfunction plays a pivotal role in the pathogenesis of CVD in T2D (12-14, 16). Particularly, impaired endothelial insulin signaling through the phosphoinositide 3-kinase (PI3K)-Akt pathway and consequent blunting of insulin-induced vasodilation and blood flow, also referred to as selective endothelial insulin resistance (14, 17), contributes to vascular disease and glycemic dysregulation (12, 16-18). Endothelial insulin resistance is the impaired ability of insulin to stimulate nitric oxide production via endothelial nitric oxide synthase (eNOS) activation through the PI3K-Akt pathway within endothelial cells, which line the inside of the vascular system and are responsible

for its functionality and health (12, 14, 16, 19, 20). Nitric oxide (NO) is a potent vasodilator and an anti-inflammatory molecule protecting against atherosclerosis and thrombosis (16, 19). Transgenic animals with an endothelial insulin receptor deletion had accelerated atherosclerosis development and more severe atherosclerotic lesions than controls (21). Cell culture experiments show impairing the PI3K-Akt pathway during insulin stimulation results in proatherogenic actions (20). Collectively, impairing endothelial insulin signaling promotes atherosclerosis, a primary contributor to CVD and mortality rates (22).

Insulin has vasodilatory actions crucial for glycemic control (11-17). Insulin induces microvascular recruitment and vasodilation to increase the delivery of insulin and glucose to skeletal muscle interstitium for glucose uptake (18). Impairing insulin-stimulated microvascular recruitment and increased blood flow with nitric oxide synthase (NOS) inhibitors impedes glucose uptake (23-28), demonstrating that the vasodilatory actions of insulin are primarily NO-dependent. Mice with genetic deletion of endothelial IRS2, the signaling protein upstream of PI3K, exhibited impaired insulin-stimulated eNOS phosphorylation, blunted microvascular recruitment, decreased interstitial insulin, and impeded skeletal muscle glucose uptake (29). These observations were recapitulated in diet-induced obese mice (29).

Isolated vessels from animal models of genetic-, and diet-induced obesity (117-120) and humans with severe obesity (29) show impaired insulin-induced relaxation. Clinical studies show the vasodilatory action of insulin is blunted by obesity and T2D during insulin infusions (30-33) and the postprandial state (34-

38), contributing to impaired glucose uptake (30, 31, 34, 35). Collectively endothelial insulin resistance and subsequently impaired vascular insulin responsiveness characterize T2D.

As endothelial insulin resistance represents a causal factor in the pathogenesis of atherosclerosis and T2D (11), there is an urgency to identify the molecular mechanisms of endothelial insulin resistance and subsequently devise effective treatments. Although the exact causal mechanisms are unclear, disruption of the PI3K-Akt pathway is a crucial contributor to endothelial insulin resistance (12, 20). Obesity, physical inactivity, glucolipotoxicity, and subsequent inflammation activate cellular stress kinases that can become overactive (17, 39). These kinases can disrupt the conserved PI3K-Akt insulin signaling pathway, present in metabolically active tissues and endothelial cells (17, 39).

One such classic stress-activated kinase is c-Jun amino-terminal kinase (JNK), whose increased activity has been shown to disrupt the PI3K-Akt pathway at the level of insulin receptor substrate 1/2 (IRS1/2) (40-42). Notably, isolated vessels from patients with severe obesity (43) and endothelial cells from patients with T2D (42) show increased JNK activity (Figure 1.1). Furthermore, the isolated vessels showed impaired insulin-induced relaxation, and the endothelial cells demonstrated impaired insulin-stimulated eNOS phosphorylation and NO-production (42). Notably, insulin responsiveness was rescued when these vessels (32) and endothelial cells (31) were treated with a JNK inhibitor. Furthermore, upregulation of JNK in endothelial cell culture impaired insulin-

stimulated eNOS activity and NO-production (31). These observations indicate JNK activity is increased in T2D vasculature and that JNK modulation may have beneficial vascular effects in T2D.

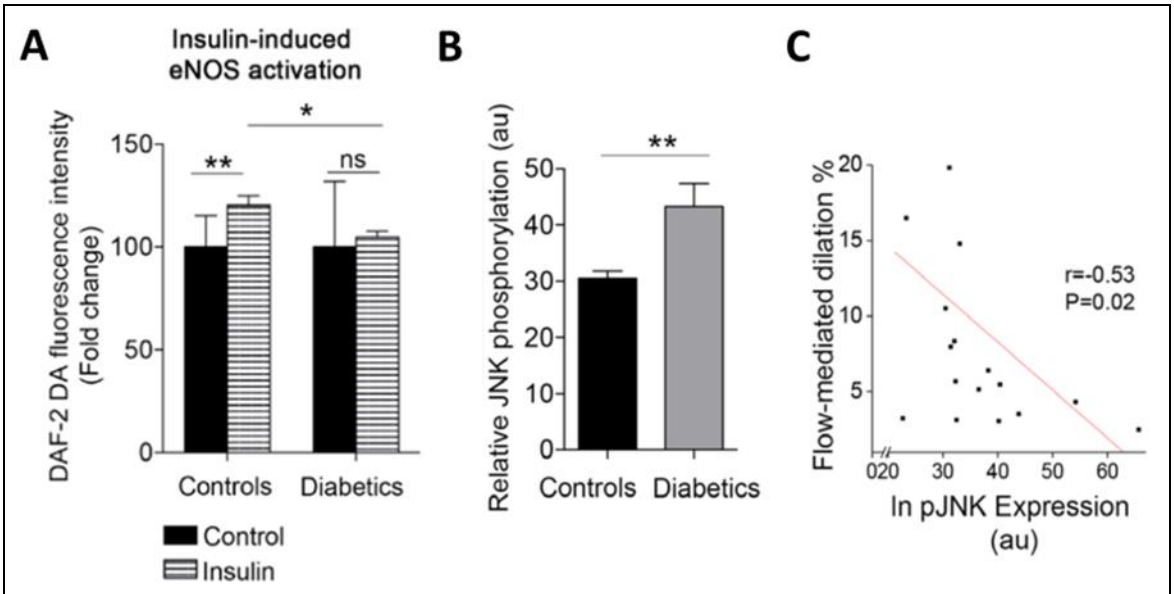

Figure 1.1 Impaired insulin-stimulated eNOS activity and increased JNK activity in endothelial cells from patients with T2D. (A) Insulin-stimulated eNOS activity and (B) JNK activity in endothelial cells collected from control subjects and patients with T2D. (C) JNK activity from endothelial cells inversely correlated with endothelial-dependent dilation as measured by flow-mediated-dilation. Adapted from Breton-Romero *et al.* (42).

Importantly, JNK activity can be regulated by heat shock protein 72 (HSP72) (44-47), an intracellular cytosolic protein chaperone molecule that can mitigate cellular stresses to maintain cellular homeostasis, in addition to its other roles that promote cellular organelle fidelity (48-50). HSP72 is highly conserved and ubiquitously expressed (48-50), including within endothelial cells (51-53). HSP72 is induced in response to cellular metabolic demands such as protein synthesis and folding, oxidative stress and injury, endoplasmic reticulum stress, hypoxia, pH changes, temperature, and exercise. Notably, intracellular HSP72 is

cytoprotective, whereas extracellular HSP72 can initiate proinflammatory signaling that promotes insulin resistance (48-50, 54). Patients with T2D have demonstrated low intracellular HSP72 in skeletal muscle and increased extracellular HSP72 expression in plasma (55). Crucially, intracellular HSP72 modulation has been implicated in developing and treating insulin resistance (48-50), making it the focus of this dissertation and hence will be referred to as "HSP72" throughout this dissertation.

Growing evidence indicate that HSP72 may be implicated in insulin resistance. Clinical studies have shown decreased HSP72 within tissues that are important for glycemic control (*i.e*., skeletal muscle, liver, and adipose tissue) from obese and insulin-resistant subjects (55-59) (Figure 1.2), which correlated with insulin sensitivity and glucose disposal during the hyperinsulinemic-euglycemic clamp procedure (56, 58). Furthermore, skeletal muscle biopsies indicate HSP72 may decline as the risk for T2D increases (58). Global genetic knockout of HSP72 has induced whole-body insulin resistance in mice (60, 61). Diet-induced insulin resistance is also associated with decreased HSP72 (57, 62, 63), including in the aorta (64).

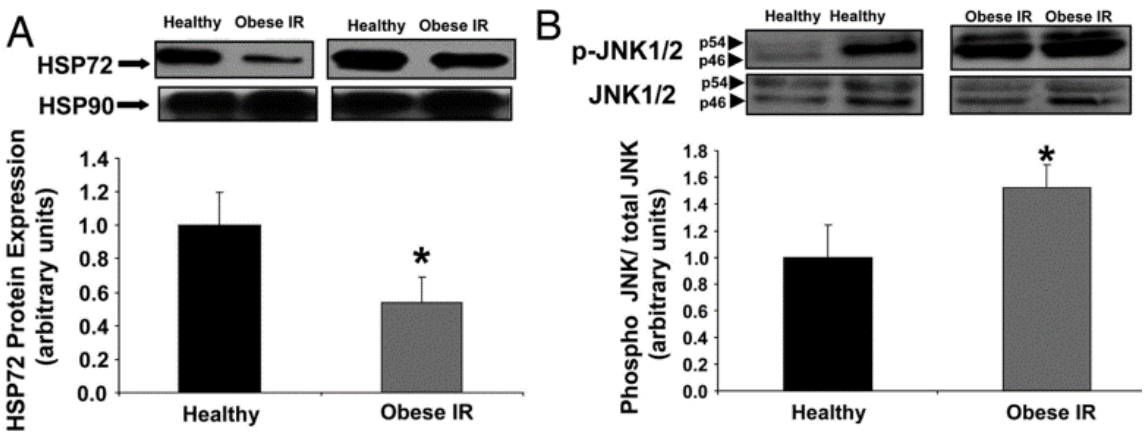

Figure 1.2 Decreased HSP72 and increased JNK activity in skeletal muscle from obese insulin-resistant individuals. (A) HSP72 and (B) JNK activity in vastus lateralis biopsies from healthy and obese insulin-resistant (IR) individuals. Adapted from Chung *et al.* (57).

Inflammatory stress kinases involved with insulin resistance, including JNK, can inhibitory phosphorylate heat shock factor-1 (HSF-1), the transcription factor responsible for the synthesis of HSP72 (65-68). Constitutive phosphorylation of HSF-1 can hold it in an inactive state under normal physiological growth conditions (67), which could explain, in part, the reduction of HSP72 in insulin resistance (50). Indeed, an inverse relationship between JNK and HSP72 has been observed in metabolically active tissues in individuals with obesity, insulin resistance, and T2D (56-59). Global knockout of HSP72 in mice has corroborated these observations (60, 61).

Crucially, increasing HSP72 via genetic manipulation (57, 61, 69, 70), exercise (62, 71), passive heating (57, 63, 64, 72-75), or mild electrical stimulation with heat treatment (76) have been shown to rescue insulin sensitivity and even prevent insulin resistance, with observed improvements in insulin-

stimulated PI3K-Akt activity (57, 61, 63, 70, 73, 76) and decreased JNK activity (57, 61, 63, 75, 76). Importantly, HSP72 suppresses JNK activity (44-46), which may explain, in part, how increasing HSP72 may improve insulin sensitivity.

Given the evidence that HSP72 could be implicated in insulin resistance and its modulation may affect insulin sensitivity, it is possible that HSP72 may be involved with endothelial insulin resistance, yet this is unknown. HSP72 could be a novel therapeutic target for treating endothelial insulin resistance in T2D. Although direct evidence is lacking, some data support the notion. HSP72 is reduced in insulin-resistant aorta (64), and JNK activity that can suppress HSP72 is elevated in endothelial cells from patients with T2D (42) and vessels from individuals with severe obesity (43). Accordingly, it was hypothesized that endothelial insulin resistance in T2D may be attributable to decreased HSP72. Conversely, increasing HSP72 may improve endothelial insulin responsiveness, perhaps by improving insulin-stimulated PI3K-Akt activity (57, 61, 63, 70, 73, 76) and inhibitory effects on JNK (57, 61, 63, 75, 76) (Figure 1.3). Therefore, it was hypothesized that increasing HSP72 could improve endothelial insulin responsiveness, in part, by reducing JNK activity.

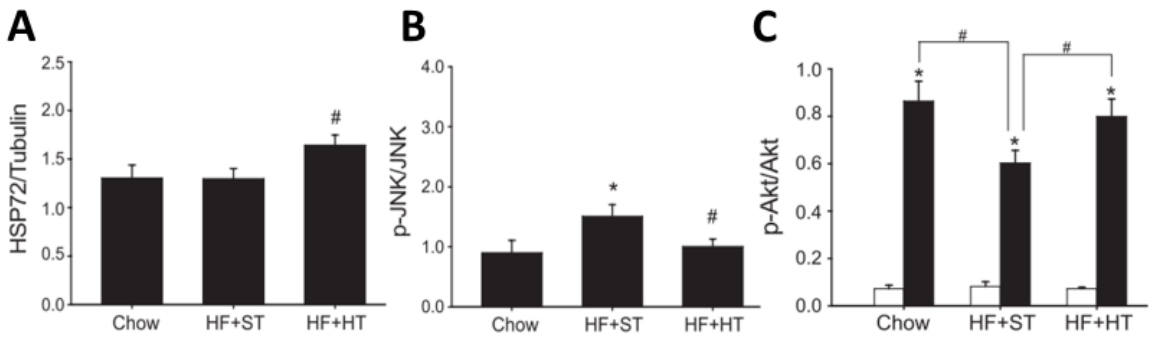

Figure 1.3 Weekly heat treatment prevented skeletal muscle insulin resistance in high-fat-diet fed rats. (A) HSP72, (B) JNK activity, and (C) insulin-stimulated Akt activity (black bars) in soleus muscle from rats after twelve weeks of chow-diet (chow), high-fat-diet with weekly sham treatment (HF+ST; 20-minute water-immersion with core temperature at 36°C), or high-fat-diet with weekly passive heat treatment (HF+HT; 20-minute water-immersion with core temperature at 41°C). In A & B, *P < 0.05 HF+ST vs. chow; #P < 0.05 HF+HT vs. HF+ST. In C, *P < 0.05 basal vs. insulin-treated; #P < 0.05 v. HF+ST. Adapted from Gupte *et al*. (63).

In line with this, it is unknown if modulating HSP72 affects endothelial insulin signaling. Silencing HSP72 has been reported to impair VEGF-stimulated Akt activity that is needed for angiogenesis (51). Furthermore, increasing aortic HSP72 with passive heating improved angiotensin 1-7 stimulated Akt-eNOS activity resulting in improved vasodilation in insulin-resistant rats (64) (Figure 1.4). Therefore, HSP72 may be necessary for endothelial processes involving Akt signaling, including endothelial insulin signaling. It was hypothesized that decreasing HSP72 impairs endothelial insulin signaling, whereas increasing HSP72 would be beneficial.

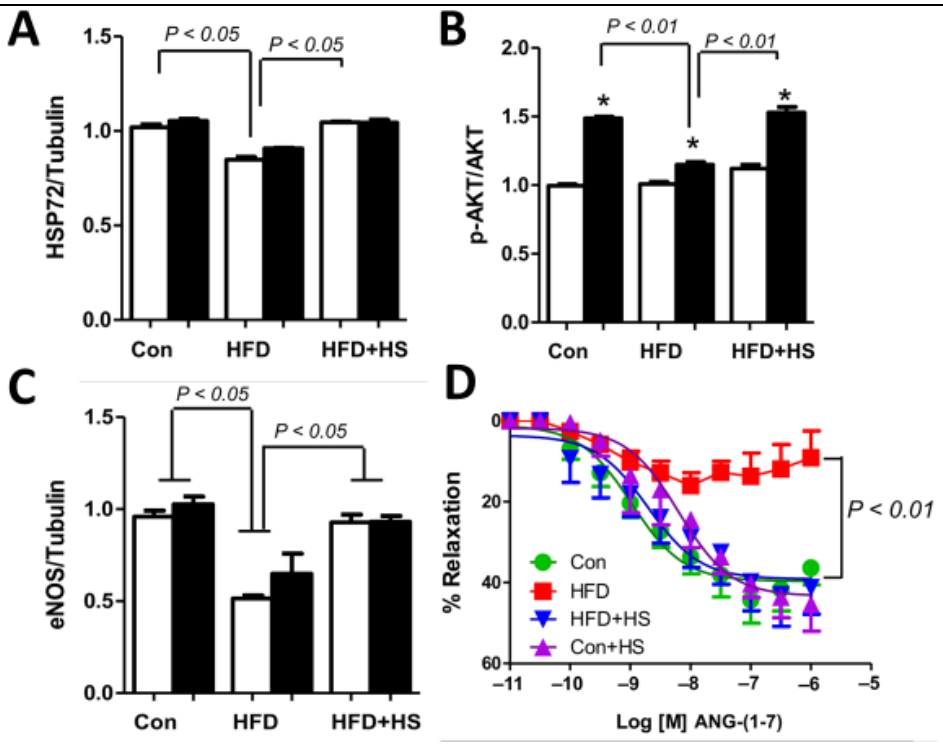

Figure 1.4 Weekly heat treatment rescues HSP72 and angiotensin (1-7) stimulated vasorelaxation in insulin-resistant rats. (A) HSP72 and (B) angiotensin (1-7)-stimulated Akt activity and (C) eNOS activity from western-blotted aortas, and (D) angiotensin (1-7)-stimulated relaxation of isolated aortic rings from of rats after twelve-weeks of control diet (Con), high-fat-diet (HFD), or high-fat diet with weekly heat treatment (HFD+HS; 20-minute blanket heating with core temperature at 41°C). For A-C, basal state, open bars; insulin-stimulated, black bars. Adapted from Karpe & Tikoo (64).

Strategies such as exercise have been shown to improve endothelial insulin responsiveness in individuals with T2D, as shown by increased insulin-stimulated blood flow (77, 78) and postprandial blood flow (79). Exercise has also been shown to upregulate and increase HSP72 expression within immune cells (80, 81) and skeletal muscle (82-91) in humans. However, individuals with T2D often have reduced cardiorespiratory fitness and a decreased capacity for sustained exercise (92-99). Furthermore, patients with T2D have often cited barriers to exercise related to physical and mental discomfort that perpetuate

unmotivated behaviors (100, 101). Therefore, alternative therapies are needed. Accordingly, passive heating has been demonstrated to upregulate and increase HSP72 expression within immune cells (102) and skeletal muscle (103-105) in humans.

Passive heating has emerged as a potentially effective therapy in treating cardiometabolic diseases. Large prospective cohort studies show that increased frequency of sauna bathing is associated with a reduced risk of fatal cardiovascular diseases and all-cause mortality (106) and CVD risk profiles (107). Chronic passive heating has improved prognosis (108) and vascular and physical functioning in vulnerable CVD populations (109-121). Chronic passive heating has been shown to improve indices of vascular function in overweight, sedentary individuals (122) and obese women with polycystic ovary syndrome (PCOS) (123), indices that have been shown to characterize T2D. Furthermore, regular passive heating has been shown to improve glycemic control in patients with T2D (124), women with PCOS (125), and sedentary overweight men (126, 127). Notably, it has been demonstrated that one hour of lower-limb heating augmented insulin-stimulated leg blood flow in healthy individuals (128). Therefore, passive heating could be used to improve endothelial insulin responsiveness in individuals with T2D. Subsequently, it was hypothesized that passive heating could improve endothelial insulin responsiveness in subjects with T2D, in part by inducing HSP72.

Accordingly, the overarching hypothesis of this dissertation was that endothelial insulin resistance in T2D is attributed to decreased endothelial

HSP72, whereas increasing endothelial HSP72 with passive heating would improve endothelial insulin responsiveness in T2D. Furthermore, the potential of HSP72 to affect endothelial insulin signaling may involve modulation of JNK activity, a stress kinase known to disrupt the PI3K-Akt pathway (shown in Figure 1.5).

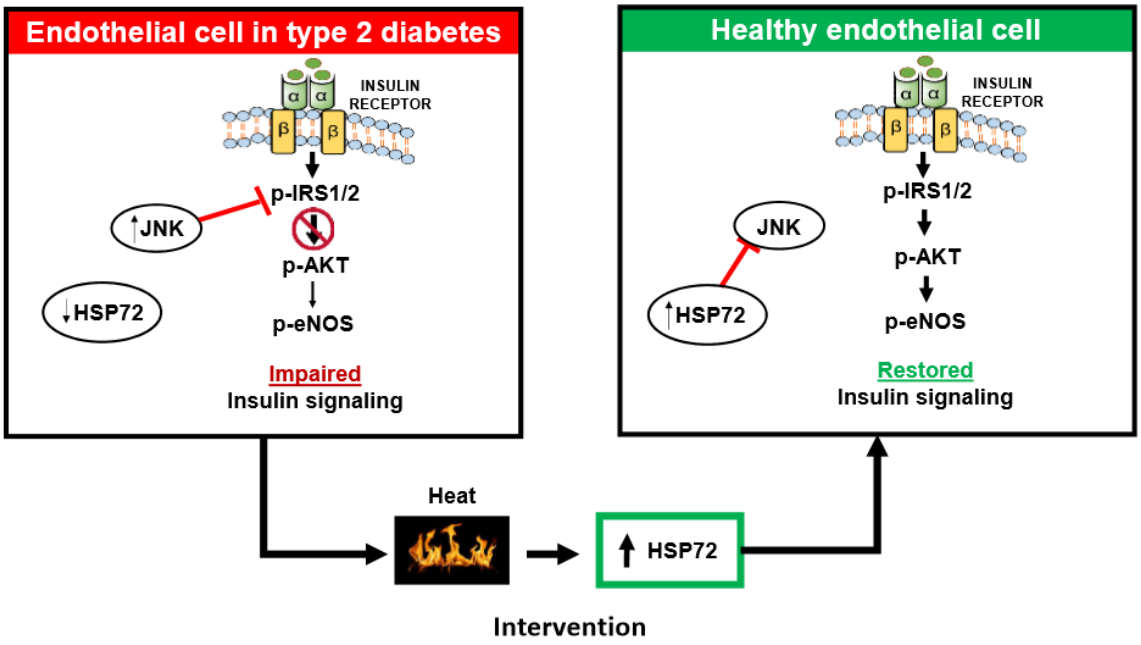

Figure 1.5 Overarching hypothesis of the dissertation. HP72 is decreased in endothelial cells in T2D (shown in the red box), promoting impaired PI3K-Akt insulin signaling via mechanisms such as increased JNK activity. Increasing HSP72 in endothelial cells by passive heating would improve PI3K-Akt signaling, in part, by decreasing JNK activity (shown in the green box).

Therefore, this dissertation accomplished the following specific aims, which are outlined below with respective hypotheses and abbreviated approaches (For detailed experimental designs and methodologies, see Chapter Three – Main Findings):

<u>Specific aim 1</u>: To determine if endothelial insulin resistance in T2D is attributed to reduced expression of HSP72.

Hypothesis: It was hypothesized that a reduced expression of HSP72 would contribute to endothelial insulin resistance in T2D.

Abbreviated approaches: The hypothesis was tested using two approaches:

Approach 1: Insulin-induced relaxation, HSP72 expression, and JNK activity of isolated aortas were compared between 20-week-old male db+ and db/db mice, a standard animal model for obesity and T2D research. It was hypothesized isolated aortas from db/db mice would exhibit blunted insulin-induced relaxation and reduced HSP72 relative to isolated aortas from db+ mice. Insulin-induced relaxation of isolated aortic rings was measured by wire myography. HSP72 expression and JNK activity of isolated aortas were measured by western blotting.

Approach 2: Postprandial leg blood flow and venous endothelial cell expression of HSP72 and JNK activity were compared between healthy subjects and subjects with T2D. It was hypothesized that subjects with T2D would display impaired postprandial leg blood flow and decreased HSP72 in collected endothelial cells relative to healthy controls. Leg blood flow was recorded in the superficial femoral artery using a 2D/Doppler ultrasound before and after a 75g oral glucose load. Postprandial leg blood flow was used as a measurement of endothelial responsiveness to endogenously produced insulin. Venous endothelial cells were collected from an antecubital vein using guidewires which

were then processed to measure HSP72 expression and JNK activity via quantitative immunofluorescence.

<u>Specific aim 2</u>: To determine if seven days of passive heating via hot water immersion improves postprandial leg blood flow with increased expression of endothelial HSP72 in subjects with T2D.

Hypothesis: It was hypothesized that, in subjects with T2D, seven days of passive heat treatment via hot water immersion to waist-level (one-hour/day) would improve leg blood flow responses to an oral glucose load (*i.e.*, endogenous insulin stimulation), and that this improvement would be accompanied with an induction of endothelial HSP72.

Abbreviated approach: Postprandial leg blood flow and venous endothelial cell expression of HSP72 and JNK activity were compared in subjects with T2D before and after seven days of passive heating. Leg blood flow and venous endothelial cell expression of HSP72 and JNK activity were collected and measured as described in specific aim 1, approach 2. The second experimental visit occurred 16-24 hours after a subject's last passive heating session. Passive heating was implemented by having a subject sit in 40.5°C water at waist level for 60-minutes per day.

<u>Specific aim 3</u>: To determine if modulation of HSP72 impacts endothelial insulin signaling in cell culture.

Hypothesis: It was hypothesized that downregulating HSP72 in endothelial cell culture would impair insulin signaling, whereas upregulating HSP72 would enhance insulin signaling.

Abbreviated approach: To test this hypothesis, cell culture experiments were conducted using human skeletal muscle microvascular endothelial cells, in which HSP72 expression, JNK activity, and insulin-stimulated Akt activation were measured via western blotting in the following experiments: 1) cells treated with small-interfering RNA to knockdown HSP72; 2) cells treated with five-days of passive heating, via an incubator set at 40.5°C, to induce and overexpress HSP72; 3) cells treated to a high glucose and palmitic acid milieu that mimics the conditions of glucolipotoxicity associated with T2D; 4) glucolipotoxic cells treated with a single session of passive heating, via an incubator set at 40.5°C, to induce and overexpress HSP72.

CHAPTER TWO – EXTENDED LITERATURE REVIEW

THE BURDEN OF TYPE 2 DIABETES AND CVD

The prevalence and incidence of type 2 diabetes (T2D) are growing in the United States and worldwide (1, 2). The number of US adults diagnosed with diabetes is expected to nearly triple by 2060, equating to over one in six adults (3). The increased rates of T2D are mirrored by the increased prevalence of obesity (4, 5) and physical inactivity (6, 7), factors known to be prime drivers of T2D (8). Notably, individuals with T2D are at increased risk of developing and subsequently dying from cardiovascular disease (9, 10).

Concurrent with the increasing prevalence of T2D is the economic burden it creates. The American Diabetes Association reported that the estimated cost of diagnosed diabetes in 2017 was $327 billion, with direct medical costs accounting for up to ~72% and the remainder attributed to reduced productivity (129). This estimated total cost increased by 26% from 2012 to 2017 and is expected to grow further (129). People with diagnosed diabetes incur average medical expenditures of ~$16,750 per year, which is ~2.3 times higher than individuals without diabetes (129). Many of these expenditures are due to comorbidities and cardiovascular-related treatments (129).

Individuals with T2D display vascular dysfunctions that manifest as hypertension, arterial stiffening, endothelial dysfunction, and impaired insulin-stimulated increases in skeletal muscle blood flow and perfusion (11). Much of these manifestations stem from complex endothelial cell mechanisms (12-14).

Notably, these vascular dysfunctions contribute to the increased risk of CVD associated with T2D (15),

Endothelial cell dysfunction plays an essential role in the pathogenesis of cardiovascular disease in T2D (12-14, 16). More specifically, impaired endothelial insulin signaling through the PI3K-Akt pathway and consequent blunting of insulin-induced vasodilation and blood flow, also referred to as selective endothelial insulin resistance (14, 17), contributes to vascular disease and glycemic dysregulation (12, 16-18). Indeed, genetic disruption of insulin signaling in endothelial cells promotes atherosclerosis (21) and limits skeletal muscle glucose uptake (29), while conversely, selective activation of the insulin receptor-PI3K-Akt signaling pathway protects against atherosclerosis formation (130). Therefore, an understanding of endothelial insulin resistance is needed to devise effective treatment strategies that reduce CVD and subsequently economic costs and mortality rates for a growing T2D populace.

ENDOTHELIAL INSULIN RESISTANCE CHARACTERIZES T2D

Insulin resistance, the hallmark feature of T2D, is characterized by a decreased responsiveness of peripheral tissues that regulate glucose homeostasis to insulin (131). Insulin resistance impedes insulin's metabolic actions that include inducing skeletal muscle glucose uptake, suppressing hepatic glucose production, and halting lipolysis from adipose tissue (131). In addition to its metabolic effects, insulin stimulates microvascular recruitment and vasodilation, increasing insulin and glucose delivery to skeletal muscle (17).

Endothelial insulin resistance is a characteristic of T2D that is observable in the early stages of the disease and aids its progression. Clinical studies have shown that insulin-stimulated blood flow and perfusion during a hyperinsulinemic-euglycemic clamp is blunted in subjects with T2D (31-33). Furthermore, endothelial insulin responsiveness declines as the risk for T2D increases, as shown by dampened insulin-stimulated blood flow and perfusion during the clamp technique in subjects with obesity (30) and first-degree relatives of subjects with T2D (132). Postprandial blood flow and perfusion, an indicator of endothelial responsiveness to endogenous insulin, has also been shown to be impaired in obesity (34-38), T2D (37), and first-degree relatives of T2D (37). Furthermore, isolated arterioles from individuals with severe obesity display suppressed insulin-induced relaxation (43). Isolated aortas and resistance arteries from animal models of genetic-, and diet-induced obesity show impaired insulin-induced relaxation (117-120), corroborating the observations in humans.

Endothelial dysfunction, classically demonstrated by impaired endothelium-dependent vasodilation mediated by nitric oxide (NO), is prominent in insulin resistance, likely due reduced ability of insulin to stimulate NO production (12, 13, 16). Additionally, micro-, and macrovascular endothelium-dependent vasodilation in response to acetylcholine and reactive hyperemia are impaired in obesity, pre-diabetes, T2D, and first degree-relatives (133). Endothelial dysfunction is clinically relevant as it can explain approximately 43% of the increase in cardiovascular mortality risk conferred by T2D (15), it can

precede observable atherosclerosis (134-137), and it can provide prognostic information (138).

Data from animal studies suggest endothelial insulin resistance could precede other detriments in T2D. Previous work using genetic models of obesity and diet-induced obesity (139-142) indicate that vascular insulin resistance is an early event in the disease process that develops prior to the manifestation of other indices of endothelial dysfunction (*e.g.*, impaired acetylcholine-induced dilation). Furthermore, insulin resistance has also been shown to occur in the vasculature before other peripheral tissues that regulate glucose homeostasis (*e.g*., skeletal muscle, adipose tissue, or liver) in diet-induced obese mice (142). Collectively, these data support that endothelial insulin resistance characterizes T2D, even occurring in the early stages of the disease.

THE VASCULAR ACTION OF INSULIN

Insulin induces microvascular recruitment of skeletal muscle for glucose uptake

The endothelial responsiveness to insulin plays an essential role in glycemic control. Insulin induces microvascular recruitment of the skeletal muscle to deliver insulin and glucose to the skeletal muscle interstitium for subsequent insulin-stimulated glucose uptake (11-17). This self-regulating insulin delivery is vital because skeletal muscle accounts for a large proportion of insulin-stimulated glucose uptake that is crucial for whole-body glycemic control (143). Furthermore, insulin delivery to the skeletal muscle interstitium has been

shown to be a rate-limiting step in insulin-stimulated glucose uptake by the skeletal muscle, occurring much slower in obese and insulin-resistant subjects (144). Insulin-stimulated microvascular recruitment promotes insulin and glucose delivery in a two-stage approach (18). Microvascular recruitment increases the exchanger interface surface area between blood supply and skeletal muscle (18). Secondly, microvascular recruitment decreases resistance at the terminal vascular beds, increasing skeletal muscle perfusion and blood flow through the feeding resistance and conduit arteries (18).

Endothelial insulin responsiveness is crucial for skeletal muscle glucose uptake

Endothelial insulin responsiveness is crucial for skeletal muscle glucose uptake. The blunted vascular insulin responsiveness that occurs in obesity and T2D, as demonstrated by reduced insulin-stimulated leg blood flow during a hyperinsulinemic-euglycemic clamp (Figure 2.1, Panel A – top), contributes to reduced leg glucose uptake (Figure 2.1, Panel A – bottom) (31). Notably, in lean individuals undergoing a hyperinsulinemic-euglycemic clamp, when N^G-monomethyl-l-arginine (L-NMMA), a nitric oxide synthase (NOS) inhibitor, was co-infused during insulin stimulation, both limb blood flow (Figure 2.1, Panel B - top) and leg glucose uptake were reduced (Figure 2.1, Panel B – bottom) (28). This impairment of insulin-stimulated blood flow accounted for ~35% reduction in skeletal muscle glucose uptake (28). Other clinical studies and animal studies corroborated the observation that impairing insulin-stimulated microvascular

recruitment and blood flow and blood flow by co-infusion of NOS inhibitors reduces skeletal muscle glucose uptake (23-27). These data collectively demonstrate that insulin-stimulated microvascular recruitment is NO-dependent.

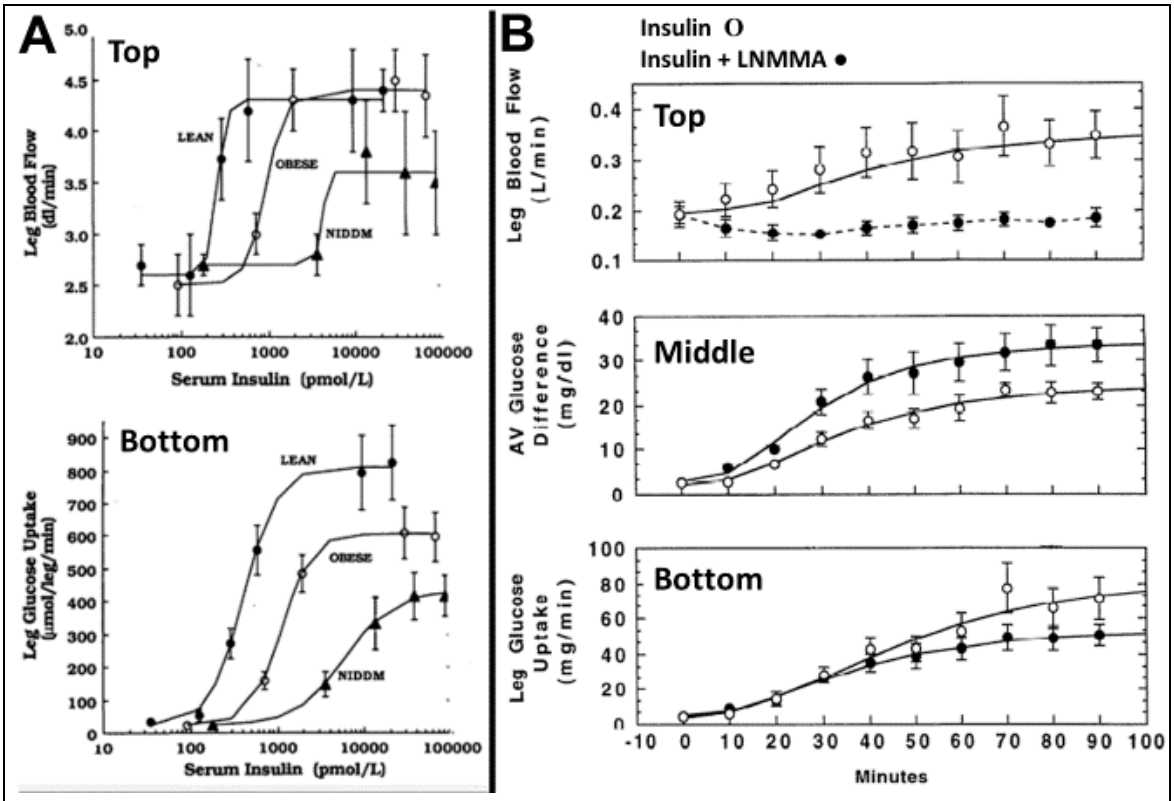

Figure 2.1 Impaired insulin-stimulated blood flow reduces glucose uptake. (A) Left panels show leg blood flow (top panel) and leg glucose uptake (bottom panel) during a hyperinsulinemic-euglycemic in lean (filled circles) and obese non-diabetic subjects (open circles) and subjects with T2D (NIDDM; filled triangles). Adapted from Laakso *et al.* (31). (B) Right panels show leg blood flow (top panel), arteriovenous glucose differences (middle panel) and leg glucose uptake (bottom panel) during hyperinsulinemic-euglycemic clamp (open circles) or co-infusion with L-NMMA (filled circles) in a group of lean healthy subjects. Adapted from Baron *et al.* (28). Layout of figure adapted from Padilla *et al.* (145).

Endothelial insulin signaling to produce NO occurs through the PI3K-Akt pathway

Insulin stimulates microvascular recruitment and increased blood flow by interacting with the endothelium and activating the endothelial insulin signaling pathway to produce NO, a potent vasodilator (17, 18). The produced NO then diffuses into adjacent vascular smooth muscle, causing relaxation of the vasculature and subsequent vasodilation-mediated increased blood flow (17, 18). As demonstrated in Figure 2.2 (145), to produce NO, insulin binds with its receptor on the endothelial membrane activating the sequential signaling cascade of insulin receptor substrate 1/2 (IRS-1/2), PI3K, Akt, and endothelial nitric oxide synthase (eNOS). eNOS then catalyzes the conversion of the substrate L-arginine to the products NO and L-citrulline (16). However, in addition to activating the PI3K-Akt pathway upon binding to the endothelial receptor, insulin simultaneously activates the RAS/mitogen-activated-protein-kinase (MAPK) pathway, producing endothelin-1 (ET-1), a potent vasoconstrictor (shown in figure 2.2). However, the overall net effect of endothelial insulin stimulation promotes vasodilation (145). The net vasodilatory effect likely occurs because NO can quash ET-1 activity and inhibit ET-1 release from endothelial cells (146-149).

In addition to NO and ET-1 regulating vascular tone, NO and ET-1 have counteracting effects that affect vascular health. NO has anti-atherogenic properties, for it reduces cell adhesion and suppresses inflammatory signaling and factor release (12, 17). In contrast, ET-1 can be considered pro-atherogenic,

as its activity promotes actions that directly oppose those of NO (as shown in figure 2.2). Based on the properties of NO and ET-1, the endothelial insulin signaling pathway has been implicated in maintaining vascular health, whereas disruption to the pathway promotes atherosclerosis (12, 14, 17, 20, 21), a mechanistic link between vascular insulin responsiveness and CVD.

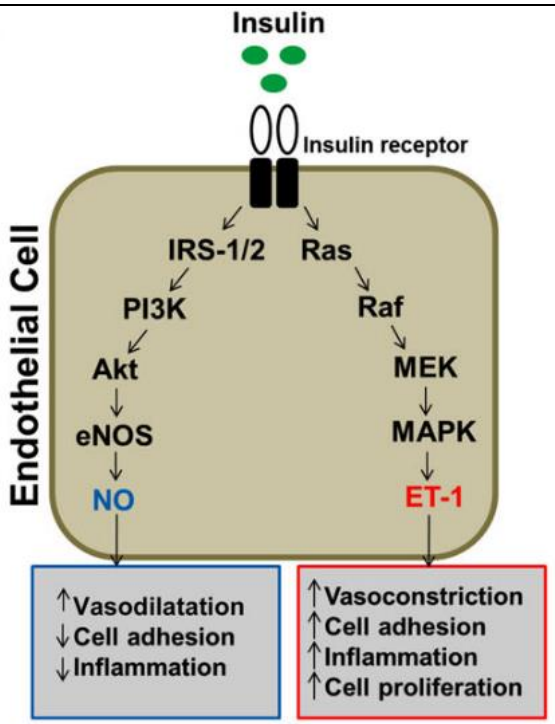

Figure 2.2 Endothelial insulin signaling pathways. In endothelial cells, insulin stimulates the divergent IRS-1/2-PI3K-Akt-eNOS pathway to produce NO (on the left) and the Ras-MAPK pathway to produce ET-1 (shown on the right). NO promotes vasodilation and anti-atherogenic actions, such as decreasing cell adhesion and inflammation. In contrast, ET-1 promotes opposing actions that promote vasoconstriction and atherogenic actions. Figure adapted from Padilla *et al.* (145).

Selective impairment of the PI3K-Akt pathway in endothelial insulin resistance

When endothelial cells become insulin resistant, there is a selective impairment of the PI3K-Akt-eNOS-NO pathway, whereas the MAPK-ET-1 axis remains intact (20, 140, 150). Some evidence demonstrates that MAPK-ET-1 activity may be increased with the selective impairment of the PI3K-Akt pathway (20, 140). Consequently, due to the PI3K-Akt pathway impairment, NO bioavailability becomes reduced, and insulin-stimulated NO production is impaired (42, 151), resulting in several complications. Firstly, impaired insulin-mediated microvascular recruitment, perfusion, and reduced glucose uptake result in hyperglycemia (30, 31, 152, 153), an independent risk factor for CVD and mediator of atherosclerosis (154). Secondly, the vasculature becomes pro-atherogenic. This phenotype occurs due to the diminished NO-mediated protection against vasoconstrictors, inflammation, cellular proliferation, platelet and leukocyte adhesion, and reactive oxygen species (ROS) (12, 13, 16).

Impairing the endothelial IRS1/2-PI3K-Akt pathway has been demonstrated to limit skeletal muscle glucose uptake (29). Mice with genetic deletion of endothelial IRS2, the signaling protein upstream of PI3K within the insulin signaling pathway, caused impaired insulin-induced eNOS phosphorylation, microvascular recruitment, decreased insulin within skeletal muscle interstitium, and skeletal muscle glucose uptake (29). These observations were recapitulated in diet-induced obese mice (29). Moreover, these impairments were reversed when insulin-induced eNOS phosphorylation

was restored by administering beraprost sodium, a stable prostaglandin (PGI)2 analog that increases eNOS expression via cyclic adenosine monophosphate (cAMP)-, protein kinase A-, and cAMP-responsive element-mediated pathways (29).

Impairing the endothelial IRS1/2-PI3K-Akt pathway has also been shown to cause atherosclerosis. Genetic deletion of the endothelial insulin receptor in mice caused atherosclerotic lesions that occurred earlier and with more severity than mice with intact insulin receptors (21). Additionally, endothelium-dependent vasodilation was impaired, and endothelial cell VCAM-1 expression increased in the deleted insulin receptor mice. Increased VCAM-1 expression promotes leukocyte adhesion to endothelial cells, a proximal step in developing neointima formation and atherosclerosis (155). Treating endothelial cells with the PI3K inhibitor Wortmannin impaired the ability of insulin to increase eNOS expression (20). However, insulin-stimulated activation of MAP kinase, Ras, and Rho proteins remained intact (20). Additionally, VCAM-1 and E-selectin expression was increased, with concomitant increased rolling interactions of monocytes with endothelial cells (20).

Conversely, selective activation of the insulin receptor-PI3K-Akt signaling pathway protects against atherosclerosis formation (130). When LDL receptor-deficient mice, a mouse model of metabolic syndrome, were treated with a drug (S597) that selectively activates the IRS-PI3K-Akt pathway and not the IRS-ERK axis, S597-treated mice had fewer atherosclerotic lesions than mice treated with vehicle or insulin injections (130). The observed reduced atherosclerosis was

due, in part, to S567 treatment slowing the accumulation of macrophages responsible for atherosclerotic lesion development and progression (130).

Collectively, these data indicate that endothelial resistance is characterized by a selective impairment of the endothelial PI3K-Akt signaling pathway, resulting in impaired skeletal muscle glucose uptake and atherosclerosis. Therefore, endothelial resistance is implicated in the pathogenesis of CVD and metabolic diseases. However, the molecular underpinnings underlying endothelial resistance in T2D remain poorly known but involve disrupting the endothelial PI3K-Akt pathway. A deeper understanding of molecular mechanisms that disrupt the PI3K-Akt pathway can help identify therapeutic strategies for preventing and treating T2D-associated vasculometabolic derangements.

Mediators of PI3K disruption

Crucially, disruption of the PI3K-Akt pathway in insulin-sensitive tissues at any point of the signaling cascade promotes insulin resistance (17, 156, 157). Although the exact mechanisms for PI3K-Akt pathway disruption are numerous and convoluted, key mediators exist (shown in Figure 2.3) (17, 156). These mediators include obesity, physical inactivity, glucotoxicity, lipotoxicity, inflammation, and their reciprocal relationships (23, 127). Each mediator can, directly and indirectly, activate mechanisms that contribute to CVD and insulin resistance (17, 156). These mediators induce oxidative stress, pro-inflammatory factors, kinases, and transcription factors (23, 127). Such pro-inflammatory

factors include tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and C-reactive protein (CRP). Such kinase and transcription factors include c-jun amino-terminal kinase (JNK), nuclear kappa factor B (NFκB), inhibitor of nuclear factor kappa-B kinase subunit beta (IKK β), and activator protein 1 (AP-1) (23, 127). Often, these kinases and factors become dysregulated, which further exacerbates chronic diseases (23, 127). Ultimately, overactive kinases and transcription factors disrupt the PI3K-Akt pathway resulting in CVD and insulin resistance.

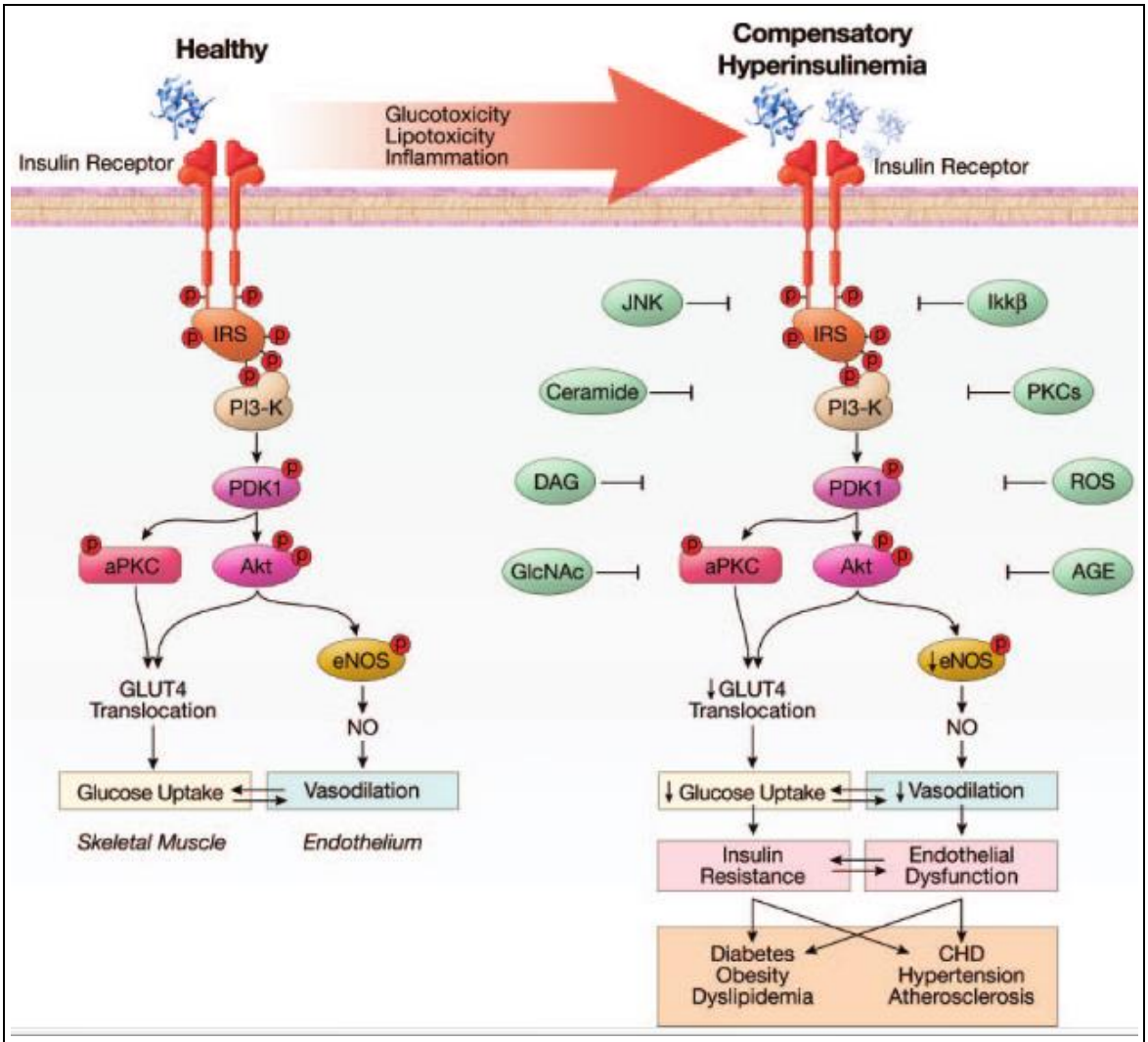

Figure 2.3 Mediators of PI3K-Akt signaling pathway disruptors with resulting consequences based on tissue type. Healthy condition (on the left), with mediators such as glucolipotoxicity and inflammation (in red arrow), inducing disruptors that inhibit the PI3K-Akt pathway at several levels (on the right) and consequences based on tissue type below. Figure adapted from Muniyappa *et al*. (17).

An overactive stress kinase that has gained much attention in the development of systemic and endothelial resistance is JNK. It has been implicated in disrupting the PI3K-Akt pathway in peripheral tissues that regulate glucose homeostasis and the vasculature (42, 43, 158-162) (as shown in Figure

2.3, specifically located as an inhibitor at the level of IRS in the pathway). JNK is a mitogen-activated protein kinase that is highly conserved and ubiquitous. The primary role of JNK activation is to appropriately coordinate signals promoting cell survival, stress tolerance, and proliferation but also signals to lead to programmed cell death (161). However, when JNK activity becomes dysregulated, JNK becomes overactive, contributing to obesity-related pathologies such as insulin resistance and T2D (158-162). A key mechanism by which JNK activation contributes to insulin resistance is that JNK activation physically disrupts the PI3K-Akt insulin signaling pathway (40, 41). JNK activation has shown to phosphorylate insulin-substrate 1 (IRS-1) at Serine 307, inhibiting the insulin receptor and IRS-1 interaction, the proximal step in insulin signaling within the PI3K-Akt pathway (40, 41) (shown in Figure 2.3 above). Therefore, overactive JNK may be a significant target for treating insulin resistance.

JNK AS A MEDIATOR OF ENDOTHELIAL INSULIN RESISTANCE

Along with the metabolic consequences of overactive JNK, activation of JNK contributes to CVD and endothelial resistance. JNK activity has been implicated in the development of atherosclerosis, with increased JNK in atherosclerotic plaque from humans and animals (163, 164). Exposing endothelial cells to high glucose (165, 166) or free fatty acids (167, 168) activate JNK, impairs endothelial insulin signaling, and upregulates the MAPK pathway resulting in the release of pro-atherogenic factors. Pharmacological inhibition of

JNK has also been demonstrated to reduce atherosclerotic plaque formation in mice (169). Furthermore, genetic deletion of JNK has been shown to reduce atherosclerosis lesions in atherosclerosis-prone apolipoprotein E knockout mice fed a high-cholesterol diet (170). Additionally, genetic deletion of JNK has been demonstrated to preserve endothelial function in high-cholesterol–fed mice (171).

JNK activity has also been implicated in endothelial resistance by impairing the endothelial insulin-PI3K-Akt pathway. JNK activity has been found to be higher in endothelial cells collected from patients with T2D when compared to non-diabetic controls (42). This higher JNK activity was associated with a low flow-mediated dilation response of the brachial artery. Both calcium ionophore-, and insulin-stimulated eNOS activation and NO-production was blunted only in the endothelial cells collected from patients with T2D. Using a specific inhibitor for JNK known as SP600125, inhibition of JNK signaling restored insulin-stimulated eNOS activation and calcium ionophore-stimulated NO production in the endothelial cells collected from patients with T2D. These observations were recapitulated in a human aortic endothelial cell culture model. Additionally, JNK inhibition with SP600125 has been shown to rescue impaired insulin-stimulated relaxation of isolated arterioles from individuals with severe obesity (43).

The observation that JNK is activated in endothelial cells from patients with T2D and is associated with endothelial dysfunction supports JNK signaling is upregulated in the T2D vasculature. Furthermore, the observation that JNK inhibition has the potential to restore insulin action in the endothelium and promote NO production suggests the possibility that JNK modulation may have

beneficial vascular effects in T2D. Non-pharmacological treatments such as exercise (172) and passive heating (57, 61) have been shown to reduce overactive JNK, in part, by increasing a JNK-regulating protein known as heat shock protein 72 (HSP72). Therefore, targeting regulators of JNK, such as HSP72, to reduce JNK activity could improve endothelial insulin-PI3K-Akt signaling and subsequently endothelial responsiveness in T2D.

HSP72 AS A THERAPEUTIC TARGET

Diminished heat shock response – contributor to diabetes pathophysiology

Overactive stress kinases such as JNK can cause insulin resistance. Therefore, insulin resistance may develop due to the dysfunctional regulation of these stress kinases. The cytoprotective heat shock response is one regulatory mechanism that mitigates overactive stress kinases, including JNK (48-50). However, overactive stress kinases, including JNK, can have an inhibitory effect on the heat shock response, promoting insulin resistance (48-50). Ultimately, a reciprocal relationship exists between stress kinases and the heat shock response that can influence insulin sensitivity (48-50).

The heat shock response is a highly conserved cellular mechanism that synthesizes protein chaperones known as heat shock proteins (HSPs) in response to cellular processes or “stressors” in order to maintain cellular homeostasis and integrity (48-50). Heat shock proteins are synthesized in response to cellular metabolic demands such as protein synthesis and folding, oxidative stress and injury, endoplasmic reticulum stress, hypoxia, pH changes,

temperature, and exercise (48-50). Overall, heat shock proteins are cytoprotective. Their functional roles include acting as molecular chaperones during protein folding, preventing protein aggregation, degrading non-functional proteins, regulating inflammatory stress kinases, and interacting with cellular organelles (such as mitochondria) to ensure physiological fidelity (48-50).

Several HSP families exist, each classified by their molecular weight. Within the HSP70 family (~70kD) is the major inducible heat shock protein 72 (HSP72; ~72kD). HSP72 is a highly conserved and ubiquitous inducible isoform encoded by the gene HSPA1A, whose intracellular expression is cytoprotective against cellular stressors (48-50). HSP72 is found in most cell types 48-50), including within endothelial cells (51-53). Specifically, HSP72 can be induced by activating the JNK and NFκB signaling pathways (48-50).

Notably, intracellular HSP72 is associated with cryoprotection, whereas extracellular expression has been associated with proinflammatory signaling (48-50, 54). It has been observed in patients with T2D that there is a divergence in the ratio of intracellular HSP72 and extracellular HSP72 content, with low intracellular expression being observed in biopsied skeletal muscle and high extracellular presence in serum (54). Indeed, extracellular HSP72 can induce proinflammatory signaling that promotes insulin resistance (54). The ratio of HSP72 between intracellular and extracellular compartments has been demonstrated to be a potential biomarker for inflammation (54).

However, HSP72 has autocrine and paracrine functions that promote anti-inflammatory adaption signaling in response to its own extracellular release (54).

Indeed, acute exercise and heating release HSP72, which induces anti-inflammatory and thermotolerance signaling over repeated exposures, resulting in an adaptative response demonstrated by less extracellular release and more intracellular expression (54). As such, HSP72 is being considered as a therapeutic exosome for diseases such as Alzheimer's (173).

A reoccurring observation within human, animal, and cell culture studies is that low intracellular HSP72 is associated with insulin resistance (48-50). Increasing intracellular HSP72 has been demonstrated to rescue insulin sensitivity and prevent insulin resistance (48-50). Hence as mentioned in Chapter 1, for this dissertation, HSP72 will be referred to within the context of intracellular expression. However, the role of extracellular HSP72 as a mediator of T2D development warrants further investigation. Regarding HSP72 acting as a cytoprotective protein, a reciprocal relationship between JNK and HSP72 has been identified in insulin resistance (48-50). Furthermore, HSP72 suppresses JNK activity (44-46), which may explain, in part, how increasing HSP72 may improve insulin sensitivity.

JNK, HSP72, and insulin resistance

The relationship between JNK and HSP72 has been demonstrated in insulin resistance. Clinical studies have shown elevated JNK activity and decreased HSP72 expression within tissues that are important for glycemic control (*i.e.*, skeletal muscle, liver, and adipose tissue) from obese insulin-resistant subjects (56-59). This inverse relationship between JNK activity and

HSP72 has correlated with insulin sensitivity and glucose disposal during the hyperinsulinemic-euglycemic clamp procedure (56, 58). Furthermore, HSP72 expression may decline as the risk for T2D increases, as shown by a progressive decrease of skeletal muscle HSP72 when compared between healthy subjects, euglycemic identical twins of subjects with glucose intolerance, subjects with glucose intolerance, and subjects with T2D (58). Animal data further corroborate the role of HSP72 in insulin resistance as genetic knockout of HSP72 increases JNK activity and induces insulin resistance (60, 61). Furthermore, diet-induced insulin resistance is also associated with decreased HSP72 (57, 62, 63), including within the vasculature (64).

A possible mechanism for the decrease of HSP72 in insulin-resistant tissues is that inflammatory kinases involved with insulin resistance can inhibitory phosphorylate heat shock factor-1 (HSF-1), the transcription factor responsible for the synthesis of HSP72 (65-68) (Figure 2.4). GSK-3, extracellular regulated kinase (ERK) and JNK, can inhibitory phosphorylate HSF-1 on serine residues 303, 307, and 363, respectively. Constitutive phosphorylation of HSF-1 on these serine residues can hold HSF-1 in an inactive state under normal physiological growth conditions (67), which could explain, in part, the reduction of HSP72 in insulin resistance (50).

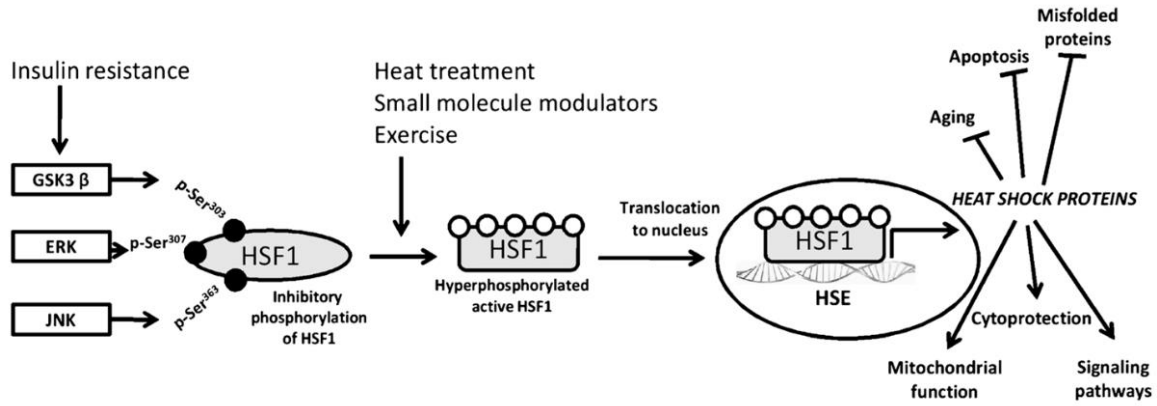

Figure 2.4 Regulation of the HSPs such as HSP72 in insulin resistance. Insulin resistance promotes activation of stress kinases GSK3β, ERK and JNK that phosphorylate Serine 303, 307, and 363, respectively on HSF-1. This inhibitory phosphorylation of HSF-1 keeps it in a repressed state. Factors such as heat treatment and exercise can hyperphosphorylated HSF-1, resulting in nuclear translocation, binding to the heat shock element (HSE), and transcription of new HSPs. HSPs then inhibit processes related to aging and apoptosis, and also enhancing signaling pathways and cytoprotection and preventing misfolding of proteins. Adapted from Geiger & Gupte (50).

HSP72 induction in insulin resistance

Notably, HSP72 can be increased in insulin resistance with stimuli such as exercise (62, 71), passive heating (57, 63, 64, 72-75), or mild electrical stimulation (76) to circumvent the insulin resistance-mediated lowered expression. However, the magnitude of HSP72 induction can be limited by insulin resistance. Atalay *et al*. (71) found that although 8-weeks of exercise training increased HSP72 levels in skeletal muscle, heart, and liver of streptozotocin-induced diabetic rats, this induction was less pronounced than observed in the nondiabetic control rats. Rogers *et al.* (62) found three days of high-fat diet feeding limited heat-mediated increases of HSP72 in low-capacity running rats. The latter study demonstrates that a deficiency in the heat shock

response, *i.e.*, the ability to synthesize HSP72, could underlie susceptibility to metabolic diseases.

HSP72 restoration improves insulin sensitivity by suppressing JNK

Crucially, increasing HSP72 via genetic manipulation (57, 61, 69, 70) or with stimuli such as exercise (62, 71), passive heating (57, 63, 64, 72-75), or mild electrical stimulation with heat treatment (76) has been shown to rescue insulin sensitivity and even prevent insulin resistance, with observed improvements in insulin-stimulated PI3K-Akt activity (57, 61, 63, 70, 73, 76) and decreased JNK activity (57, 61, 63, 75, 76). Importantly, HSP72 suppresses JNK activity (44-47), which may explain, in part, how increasing HSP72 may improve insulin sensitivity.

It has been demonstrated that HSP72 suppresses JNK by inhibitory binding. Park *et al.* (47) showed that HSP72 could modulate JNK by directly binding to it. Both pre-treatment with mild heat shock (43°C for 20 minutes) and constitutive overexpression of HSP72 suppressed ultraviolet irradiation-induced JNK activity in NIH 3T3 cells. In contrast, HSP72 silencing abolished the suppressive effect of mild heat shock. The suppression of UV- irradiation-induced JNK activity is notable as this form of stimuli does not cause protein damage (45). Indeed, HSP72 has been shown to inhibit JNK activity induced by both protein-damaging and non-damaging stimuli (44, 45). Park *et al.* (45) performed in vitro and kinase studies indicating HSP72 bound to the isoform JNK1, with the peptide-binding domain of HSP72 being crucial to the binding to

and inhibition of JNK1. Furthermore, in vivo binding of endogenous HSP72 to JNK1 in NIH 3T3 was confirmed by co-immunoprecipitation (47).

Collectively, all these data support the notion that a reciprocal relationship exists between JNK and HSP72. This relationship could be targeted to treat insulin resistance, increasing HSP72 mitigating JNK activity to improve insulin sensitivity.

HSP72 & THE VASCULATURE – A NOVEL TARGET FOR ENDOTHELIAL INULIN RESISTANCE

Given the evidence that HSP72 could be implicated in insulin resistance and its modulation may affect insulin sensitivity, HSP72 may be involved with endothelial insulin resistance, yet this is unknown. HSP72 could be a novel therapeutic target for treating endothelial insulin resistance in T2D. Although direct evidence is lacking, other data exists that could be extrapolated to support the notion that reduced expression of HSP72 contributes to endothelial insulin resistance. Firstly, HSP72 has been shown to be reduced in isolated aortas from insulin-resistant rats (64). Secondly, JNK activity that can suppress HSP72 is elevated in endothelial cells from patients with T2D (42) and vessels from individuals with severe obesity (43). Accordingly, it was hypothesized that endothelial insulin resistance in T2D is attributable to decreased HSP72. Conversely, increasing HSP72 may improve endothelial insulin responsiveness, perhaps by its ability to improve insulin stimulated PI3K-Akt activity (57, 61, 63, 70, 73, 76) and inhibitory effects on JNK (57, 61, 63, 75, 76). Therefore, it was

hypothesized that increasing HSP72 could improve endothelial insulin responsiveness, in part, by reducing JNK activity.

In line with this, it is unknown if modulating HSP72 affects endothelial insulin signaling. Silencing HSP72 has been reported to impair VEGF-stimulated Akt activity that is needed for angiogenesis (51). Furthermore, increasing aortic HSP72 with passive heating improved angiotensin 1-7 stimulated Akt-eNOS activity resulting in improved vasodilation in insulin-resistant rats (64). Therefore, HSP72 may be necessary for endothelial processes involving Akt signaling, including endothelial insulin signaling. It was hypothesized that decreasing HSP72 impairs endothelial insulin signaling, whereas increasing HSP72 would be beneficial. Ultimately, upregulating HSP72 in endothelial cells could improve endothelial insulin responsiveness in patients with T2D. Beyond a potential role of HSP72 mitigating endothelial insulin resistance, targeting endothelial HSP72 may improve other vascular dysfunctions that characterize individuals with T2D.

HSP72 protects against atherosclerosis and injury due to ischemia

HSP72 has been demonstrated to protect against arterial calcification and inward vascular remodeling and stiffening, precursors for CVD, atherogenic lesion rupture, and hypertension. Arteries from patients with coronary artery disease and chronic kidney disease have shown low expression of HSP72 (174). Human aortic vascular smooth muscle cells (HA-VSMCs) grown in high calcium media to induce calcification recapitulated the observations from the patient arteries (174). Furthermore, silencing HSP72 in HA-VSMCs induced calcification

(174). Notably, calcified HA-VSMCs chronically heat-treated (43°C for 30 minutes per day, for 21 days) robustly increased HSP72 expression and reduced calcification (174). In rodents with implanted polyethylene tubes around femoral arteries to induce intimal thickening, rodents treated with daily heated water immersion (40.5-41.5°C, for 15 minutes) had increased vascular HSP72 and attenuated neointimal thickening and reduced inflammatory cell infiltration into the adventitia (175). These data collectively indicate that HSP72 protects against vascular calcification and could protect against CVD within T2D.

Increasing HSP72 may improve CVD event survival in individuals with T2D, who are more susceptible to myocardial infarction and reoccurring events (176). Increasing HSP72 via passive-heating has been shown to protect ventricular and endothelial function after ischemia-reperfusion injury (177). Furthermore, increasing HSP72 via mild heating has been shown to protect against ischemia-reperfusion injury applied to the cerebral vasculature (178). Notably, HSP72 has protected myocardial cells, cerebral vasculature, and neurons from ischemia-reperfusion injury by suppressing JNK activity (178, 179).

HSP72 protects against hypertension

HSP72 has been shown to protect against hypertension. Crosstalk exists between HSPs and the renin-angiotensin-system in hemodynamic regulation. HSP72 has been demonstrated to be upregulated in response to Angiotensin II (Ang II), a classic vasoconstrictor, as Ang II stimulates multiple signaling pathways related to cellular injuries, such as NF-κB (180). Rodents pretreated

with a single heating session via heated blankets to maintain core temperature at 41°C for 15 minutes were protected against two weeks of Ang II-induced hypertension (180). The heat-mediated suppression of Ang II-induced hypertension was associated with increased aortic HSP72 and HSP27, decreased aortic IL-6, and inhibition of NF-κB and p65. Furthermore, the prior heat treatment suppressed two weeks of norepinephrine-induced hypertension (180).

Increased HSP72 is associated with improvements in vascular relaxation in the presence of insulin resistance

In the only study so far examining vascular HSP72 in insulin resistance, rodents fed a twelve-week high-fat diet (HFD) that resulted in obesity and insulin resistance had reduced aortic HSP72 and blunted Angiotensin-(1-7)-stimulated vasorelaxation of isolated aortas (64). Angiotensin (1-7), acting through receptor Mas (G-protein–coupled), counter-regulates the actions of Ang II (64). Notably, when HFD-fed rats received concurrent heat treatment via weekly blanket heating to maintain the core temperature at 41°C for 20 minutes, aortic HSP72 expression and Angiotensin-(1-7)-stimulated vasorelaxation was rescued, and obesity and insulin resistance was prevented (64).

HSP72 protects angiogenesis

Angiogenesis promotes new blood vessels formation and vessel growth needed for tissue repair and recovery after ischemic injury (181). HSP72 may be

necessary for angiogenesis to occur. Endothelial cell culture experiments showed pharmacological inhibition of HSP70s with KNK437, and siRNA-mediated knockdown of HSP72 blunted vascular endothelial growth factor (VEGF)-stimulated Akt/eNOS signaling, which attenuated angiogenesis (51). These observations were recapitulated in vivo, in which five days of KNK437 treatment attenuated recovery from a unilateral ischemic hind limb model (51). These data indicate that HSP72 is important for endothelial processes involving Akt signaling. HSP72 could be essential for other endothelial signaling processes, such as insulin-stimulated activation of Akt, yet this remains uninvestigated.

Suppressed angiogenesis also impairs wound healing in diabetes which can cause other potentially fatal complications such as sepsis (182). HSP72 has been shown to be reduced in wound lesions from diabetic and hypercortisolemic states (183). Notably, passive heating (184, 185) and upregulating HSP72 (186) have been demonstrated to improve diabetic wound healing.

PASSIVE HEATING – A POTENTIAL THERAPY

Regular passive heating reduces the risk for chronic disease and has clinical applications

As noted in the previous sections, passive heating has been shown to induce HSP72 and improve insulin resistance and cardiometabolic disease states in animal studies and cell-culture experiments. Subsequently, passive heating has been examined as a potential clinical therapy for humans. Highlighting the clinical relevance of regular passive heating, a prospective

longitudinal study of 2315 middle-aged men found sauna bathing more than once a week reduced the risk for cardiovascular- and all-cause mortality (106). This decreased risk was further augmented when sauna bathing occurred 4-7 times a week and with sessions lasting more than 19 minutes (106). Other prospective studies have indicated that regular passive heating reduces CVD risk factors (107) and reduces the risk for other diseases such as stroke (187), acute and chronic respiratory disease (188), dementia and Alzheimer's disease (189), and systemic inflammation indicated by CRP levels (190).

Notably, chronic passive heating has shown to be effective in treating vulnerable clinical populations. Passive heating has been demonstrated to improve the prognosis of patients with chronic heart failure (108), and both vascular outcomes and physical functioning in patients with coronary artery disease (116), chronic heart failure (109-115), and peripheral arterial disease (117-121).

Cardiovascular responses during passive heating

During passive heating, there are coordinated responses of the cardiovascular system to dissipate heat. Cutaneous vasodilation to increase blood flow to the skin for heat dissipation is the hallmark response during heating, which is coordinated by neural and local mechanisms (191). In response to heating, there is a release of multiple neurotransmitters that stimulate cutaneous vasodilation, including acetylcholine, NO, vasoactive intestinal peptide, substance P, and prostaglandins (191). Local mechanisms

promote vasodilation in a two-stage manner. Firstly, vanilloid type 1 receptors in afferent cutaneous sensory nerves sense increased temperature and release vasodilatory neurotransmitters (191). Subsequently, endothelial HSP90 and eNOS interact, producing NO that diffuses into adjacent vascular smooth muscle (191).

In response to the cutaneous vasodilation during heating, there is an increase in cardiac output and coordinated reduction of blood flow to the splanchnic and renal circulations (191). These responses support increased blood flow to the skin and maintenance of blood pressure despite a reduction in peripheral vascular resistance (191). With these changes in cardiovascular hemodynamics, blood flow and anterograde shear rate increase through the conduit and peripheral arteries (191). Notably, increased blood flow and anterograde shear rate increase the shear stress the endothelium experiences (192). Endothelial shear stress is the tangential stress derived from the friction of the flowing blood on the endothelial surface of the arterial wall (192). Increased endothelial shear stress is a potent stimulus for inducing NO-production through mechanotransduction signaling that upregulates eNOS activity and promotes antiatherogenic protection (193).

Changes in the autonomic nervous system coordinate most responses during passive heating. Specifically, passive heating increases sympathetic nervous system activity and promotes parasympathetic withdrawal to coordinate redistribution of blood flow to the skin, increased cardiac output, and increased non-cutaneous vascular resistance to maintain blood pressure (191). The

sympathetic nervous system also coordinates sweating to promote cooling off in response to heating (194).

Passive heating improves vascular dysfunctions that characterize T2D

Individuals with T2D often exhibit vascular dysfunctions such as hypertension, arterial stiffening, and endothelial dysfunction (11). Notably, passive heating has been demonstrated to improve these vascular dysfunctions that characterize T2D. Eight weeks of hot water immersion (40.5°C water, 60-minutes/session, 3-5 sessions/week) improved brachial artery flow-mediated dilatation and reduced arterial stiffness, blood pressure, and carotid intima-media thickness in overweight, sedentary subjects (122). These findings were recapitulated in obese insulin-resistant women with polycystic ovary syndrome (PCOS) (123). Additionally, this passive heating model decreased resting sympathetic activity in women with PCOS, which is clinically relevant as increased resting sympathetic activity is associated with vasoconstriction, hypertension, obesity, and insulin resistance (195, 196).

Brunt *et al.* (197) have conducted one of the few mechanistic studies in humans to determine if improvement in microvascular function after eight weeks of hot water immersion was NO-dependent. Before and after the intervention, an arm was challenged with local heating. During the heat challenge, vascular conductance was measured via laser doppler during microdialysis infusions of either lactate ringer (as control), NOS inhibitor, or Tempol (a superoxide dismutase mimetic to reduce oxidative stress). After the eight-week intervention,

NOS infusion decreased vascular conduction, whereas conductance was not different in either the control or tempol infusion. These data indicate that improvements in microvascular function following eight weeks of passive heating were NO-dependent.

The beneficial effects of passive heating on the vasculature are evident after an acute session, which has been found to reduce arterial blood pressure (118, 198), arterial stiffening (118), and improve microvascular function (199). Notably, blood pressure was reduced in subjects with T2D after an acute bout of clavicle level hot water immersion (40°C water, for 1 hour) (200). An acute session of lower-body heating reduced circulating ET-1 in healthy participants (105), indicating that passive heating could reduce circulating vasoconstrictive and proatherogenic peptides associated with selective endothelial insulin resistance.

The impact of acute passive heating on FMD responses has been equivocal. Romero *et al.* (199) found that lower leg hot water immersion improved femoral artery FMD responses in older subjects but not in young adults. Thomas *et al.* (198) found no improvement in femoral artery FMD in healthy individuals following lower body hot water immersion or treadmill running. Brunt *et al.* (201) found no improvement of brachial artery FMD after clavicle level hot water immersion in healthy subjects but did see that the heating session protected FMD responses in a model of ischemia-reperfusion injury.

The observation that passive heating can be protective against induced vascular dysfunction was corroborated by Teixeira *et al.* (202), who showed that

passively heating the lower limbs three times a day for 30-minutes prevented impaired popliteal artery FMD responses following five days of reduced physical activity. Additionally, Restaino *et al.* (203) demonstrated that lower leg heating could mitigate popliteal artery endothelial function impairments due to three hours of prolonged sitting. Collectively, passive heating can prevent endothelial dysfunction in the presence of sedentary activity. Notably, passive heating induces vascular responses and adaptions that reduce the cardiovascular risk profile. A visual summary of the effect of acute and chronic passive heating on the cardiovascular system is provided in Figure 2.5 (below).

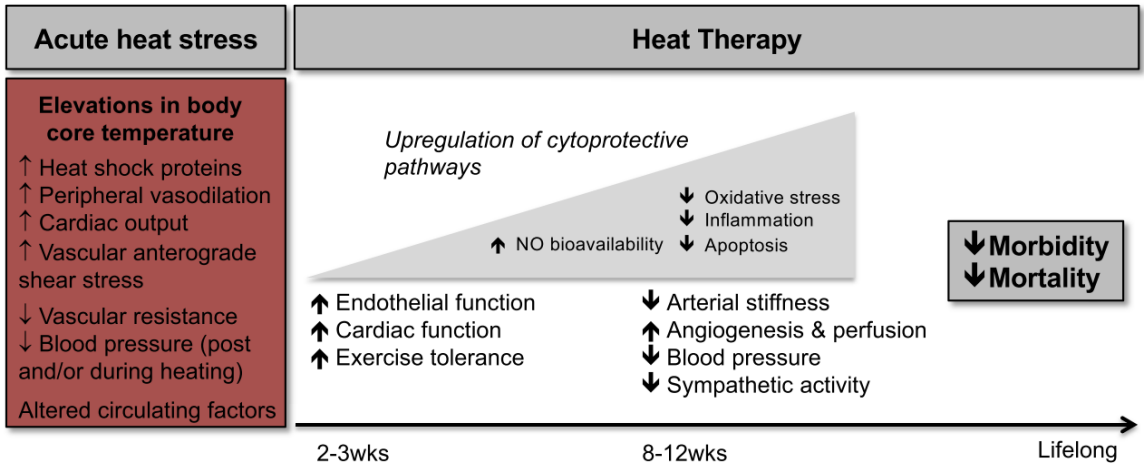

Figure 2.5 Summary of how heat stress can lead to short-term, long-term, and lifelong reductions in cardiovascular risk when repeated in the form of heat therapy. Figure adapted from Brunt & Minson (204).

Modality of passive heating can impact effects on the cardiovascular system

Modality of passive heating may also play a role in improved vascular outcomes. Water immersion has been suggested to be more beneficial for the

cardiovascular system than other passive heating modalities (such as saunas, Waon therapy) because it exerts hydrostatic pressure on the body (204). Water immersion can exert hydrostatic pressure on the body that can have independent effects on effects on cardiovascular function, such as by increasing cardiac output (205, 206), mean arterial blood pressure (206), conduit artery diameter (206), and arterial compliance (205).

Passive heating improves insulin resistance and glycemic control

Chronic passive heating has been shown to help treat insulin resistance. Hooper's seminal work showed that three weeks of hot water immersion (37.8°C to 41.0°C water, 30 minutes per session, 6 days per week) lowered body weight, fasting plasma glucose level, and glycosylated hemoglobin in patients with T2D (124). Ely *et al.* (115) showed that eight weeks of hot water immersion (40.5°C water, 60-minutes/session, 3-5 sessions/week) lowered plasma glucose levels in response to a 75g OGTT in women with PCOS, a highly insulin-resistant population. Furthermore, fat biopsies showed passive heating improved insulin-stimulated Akt activity (125). Hoekstra *et al.* (126) showed that two weeks of hot water immersion (39°C water, 60-minutes/session; 10 sessions) reduced fasting glucose and insulin concentrations in sedentary overweight men. Pallubinsky *et al.* (127) showed ten consecutive days of passive heating, whereby participants stayed in a “warm chamber” (ambient temperature of 34.4 ± 0.2°C) for 4-6 hours per day, improved insulin sensitivity and glucose disposal in overweight men in response to a hyperinsulinemic-euglycemic clamp. Collectively, chronic passive

heating can improve insulin sensitivity, but the exact mechanisms remain unclear and need further interrogation.

Passive heating mediated improvements in glycemic control are likely due to adaptation from repeated exposure rather than acute bouts. Fasted glucose levels in sedentary overweight men were not lowered when measured at one or two hours after a single session of hot water immersion (39°C water, 60-minutes). In subjects with T2D, acute hot water immersion (40°C water, 60-minutes) did not improve postprandial glycemic control in response to an oral glucose load ingested an hour after immersion (200). Furthermore, in healthy subjects or subjects with T2D, a single-session of hot water immersion (39.4°C water, 60-minutes) did not improve postprandial glycemic control in response to a glucose challenge ingested twenty-four hours after immersion (207).

Does induction of HSP72 in humans contribute to improved glycemic control?

Exercise has been shown to upregulate and increase HSP72 within immune cells (80, 81) and skeletal muscle (82-91) in humans. Similarly, this has been recapitulated by passive heating that has increased HSP72 expression within immune cells (102) and skeletal muscle (103-105) within individuals. An increase in core temperature via exercise and heating has often been cited as a primary stimulus for increasing HSP72 expression (204). Gibson *et al.* (80) showed that increasing HSP72 mRNA in immune cells of exercising humans depended on increasing core temperature, with more robust induction at ~38.5°C

and the longer this core temperature was maintained. Based on these observations, clinical studies aiming to increase HSPs to improve insulin sensitivity and cardiovascular risk profiles have increased core temperature to 1-1.5°C (122, 123, 125). Notably, isolated limb heating with unilateral water perfused pants increased HSP72 mRNA in skeletal muscle without increases in core temperature (105), therefore increased temperature and not specifically increased core temperature may be a requirement to increase HSP72.

Despite recorded increases in core temperature, clinical studies examining whether passive heating increased HSP72 and improved glucose homeostasis found improved insulin sensitivity and glycemic control without any detected change in HSP72 in biopsied adipose tissue (125), skeletal muscle (127), or collected immune cells (126). These findings contrast those observed in animal studies showing passive heating improves insulin sensitivity and glycemic control due to increasing HSP72 (57, 61, 63, 70, 72-75). A possible discrepancy between animal and human data could be that animals are safely exposed to higher temperatures than human subjects. It should be noted that detection of increased HSP72 following acute exercise or heating has varied across human studies ranging from no change (208) to being detected forty-eight hours later (83) and even staying detectable seven days after the session (83).

More clinical studies are needed to examine the mechanisms for passive heat-mediated improvements in glycemic control. Of the studies performed so far, Ely *et al.* (125) has provided the most mechanistic insight, demonstrating that chronic passive heating improved insulin-stimulated Akt signaling of biopsied

adipose tissue from women with PCOS. One potential mechanism not fully explored is a potential contribution of heat-induced increases of blood flow to skeletal muscle (112).

Heat-induced improvements in glycemic control could be due to actions from the vasculature

After observing that three weeks of hot water immersion improved glycemic control in patients with T2D, Hooper hypothesized that this improvement occurred because of repeated heat-induced increases of blood flow to the skeletal muscle promoting nutrient delivery (124). Indeed, skeletal muscle blood flow can independently modulate insulin-dependent glucose uptake, augmenting insulin-mediated glucose uptake (209). Delivery of insulin and glucose to the skeletal muscle interstitium is vital for skeletal muscle glucose uptake, which relies heavily on endothelial insulin responsiveness (29). Some support for Hooper's hypothesis comes from observations that passive heating can increase blood flow to the skeletal muscle during heating. Walsh *et al.* (128) demonstrated with single limb heating in 40-42°C water that skeletal muscle blood flow (Figure 2.6, panel B) and perfusion (Figure 2.6, panel C) are increased during immersion.

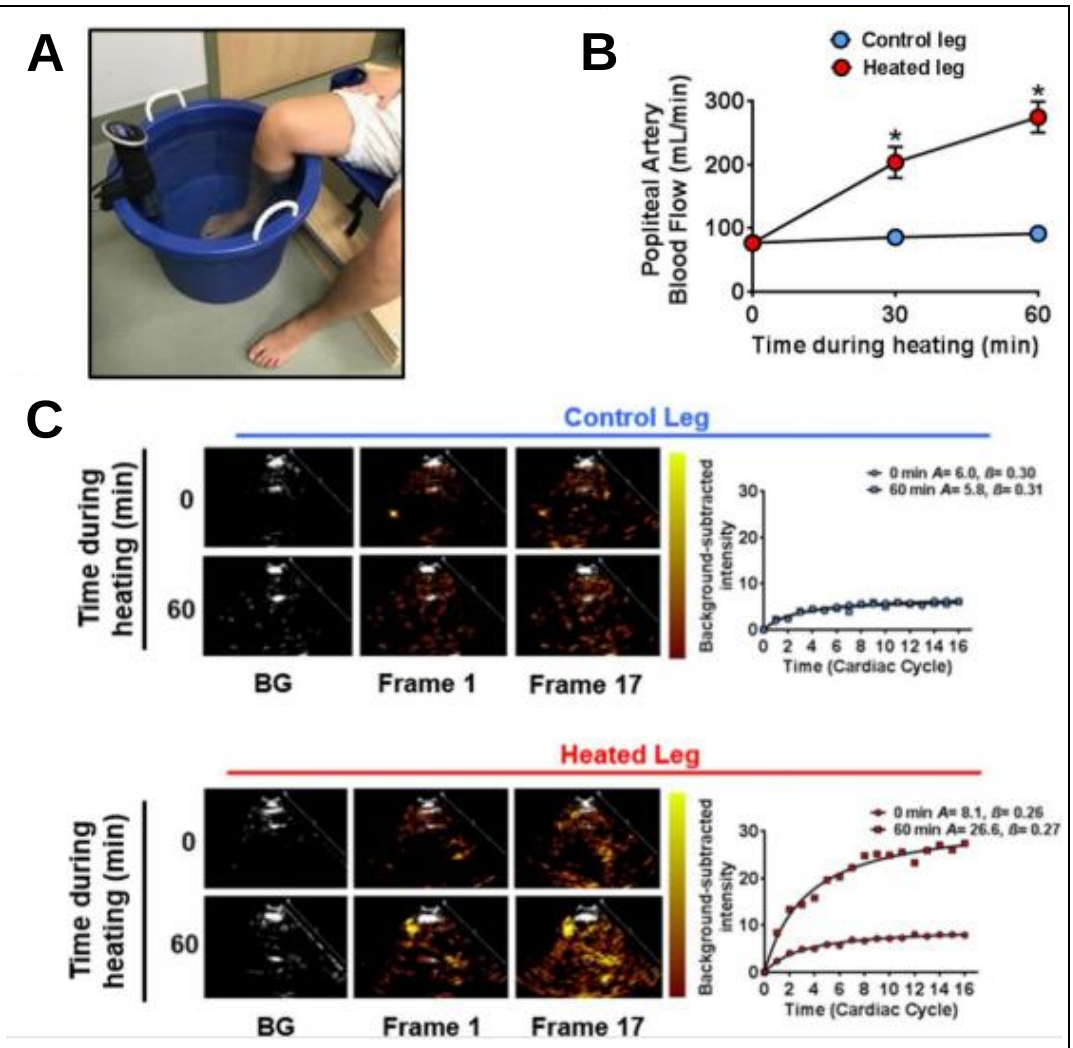

Figure 2.6 Popliteal artery blood flow and lower leg skeletal muscle perfusion are increased during hot water immersion. (A) Experimental set up during single-leg heating. (B) Popliteal artery blood flow and (C) calf muscle perfusion were increased in the heated leg only during hot water immersion. *P<0.05 vs. control leg. Figure adapted from Walsh *et al.* (128).

Whether this increased blood flow from heating promotes glucose uptake has not been evaluated. Nonetheless, a critical observation from the study by Walsh *et al.* (128) was that in healthy subjects, this hot water immersion subsequently augmented lower leg insulin-stimulated blood flow (Figure 2.7, panel A) and insulin-stimulated skeletal muscle microvascular recruitment (Figure

2.7, panel B) and perfusion (Figure 2.7, panel C) compared with the unheated control leg (128).

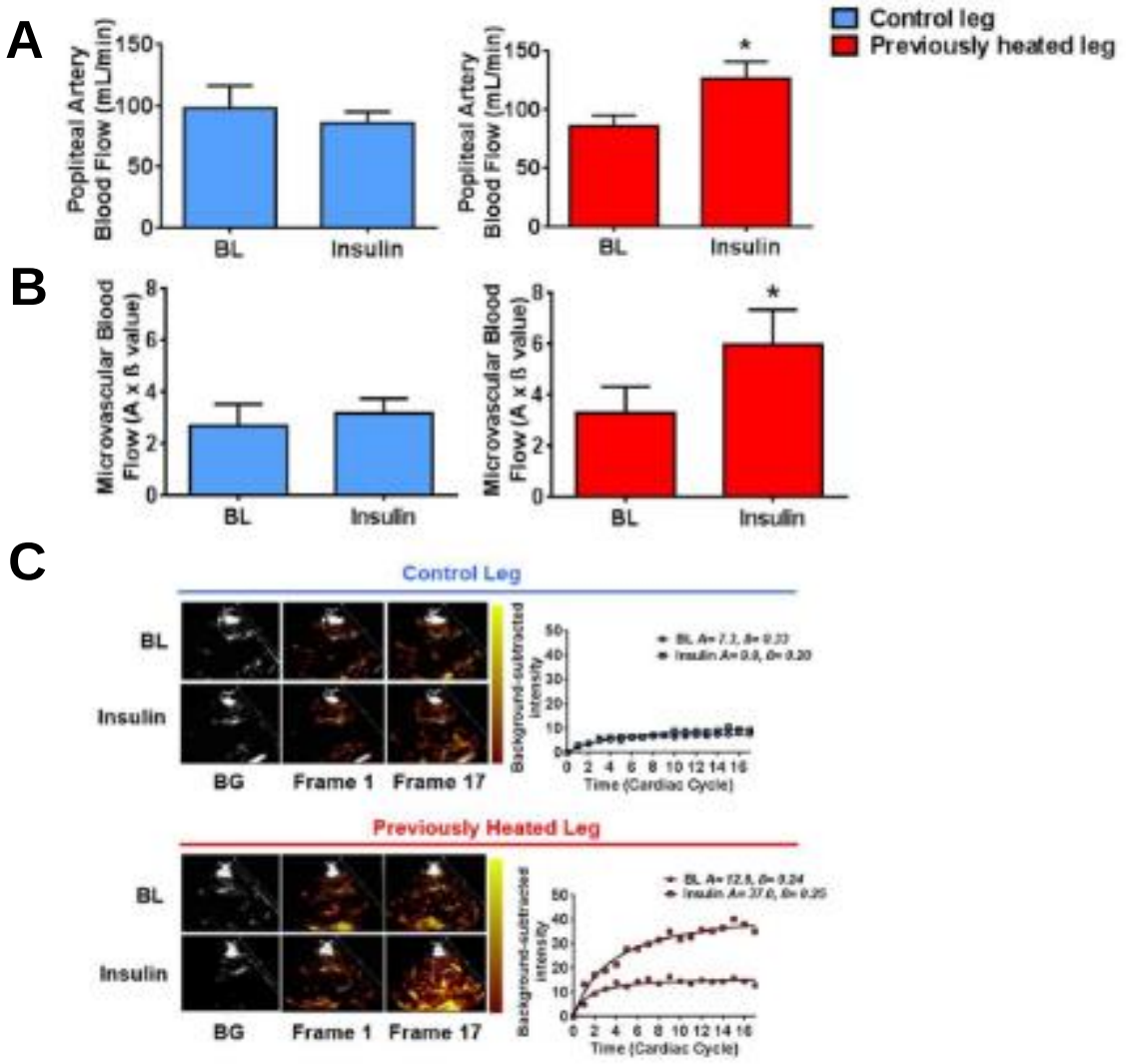

Figure 2.7 One hour of passive heating via hot water immersion augmented insulin-stimulated blood flow. (A) Popliteal artery blood flow, (B) microvascular blood flow, and (C) perfusion of the calf muscle was increased during a hyperinsulinemic-euglycemic clamp in the previously heated leg, relative to the control leg. Adapted from Walsh *et al.* (128).

Passive heating has insulin-sensitizing effects on the vasculature that could improve endothelial insulin responsiveness in patients with T2D

Based on the finding by Walsh *et al.* (116) that passive heating can augment endothelial insulin sensitivity, it can be postulated that passive heating could be used to improve endothelial insulin responsiveness in patients with T2D. However, the mechanism for this improvement is not yet fully explored. Walsh *et al.* (128), reported that insulin stimulation occurred one hour after hot water immersion had ended. HSP72 protein content has been shown to increase an hour after heat exposure in the soleus muscle of rats (210) and in human peripheral mononuclear cells (211). Although speculative, it is possible that HSP72 was increased in endothelial cells due to the heating intervention used by Walsh *et al.* (128). Furthermore, if HSP72 was increased, it is unknown if it contributed to improved insulin sensitivity through such mechanisms as reducing stress kinase activity. If so, this phenomenon could benefit patients with T2D. All of which has yet to be investigated but could provide novel therapeutic evidence in humans.

Little work has been conducted on evaluating the time course for HSP72 induction in endothelial cells. In bovine aortic endothelial cell culture, Harris *et al.* (223) showed that exposure to 42°C for one hour did not increase HSP72 when assessed twenty-four hours later, however, when cells were exposed to 45°C, HSP72 was increased. In contrast, when rats were heat-treated at a core temperature of 42°C for fifteen minutes, HSP72 expression was found to be increased in the aorta twenty-four hours after the heating session. This study

demonstrated that increased HSP72 could be observed twenty-four hours after the last heating session, implying HSP72 mediated improvements may take effect much later on. Therefore, repeated bouts of passive heating over time may accumulate increased HSP72 resulting in improvements.

Induction of HSP72 is not the only mechanism by which passive heating has insulin-sensitizing effects on the vasculature. The finding by Walsh *et al.* (128) that acute passive heating augmented insulin-stimulated blood flow in healthy participants was part of a series of experiments examining the role of endothelial shear stress as being a primary stimulus for exercise-mediated improvements in endothelial responsiveness. Exercise has been shown to improve insulin-stimulated blood in individuals with T2D in some studies (77-79), but not all (33).

Exercise improves endothelial insulin responsiveness

Exercise has also been shown to be effective for improving endothelial responsiveness. Dela *et al*. (77) showed 10-weeks of single-legged aerobic exercise, improved insulin-stimulated leg blood flow of the trained leg compared to the untrained leg during a hyperinsulinemic-euglycemic clamp in healthy subjects and subjects with T2D. Furthermore, the improvements in insulin-stimulated blood flow were associated with increased leg glucose uptake in the exercise-trained limb (77). Similarly, Holten *et al.* (78) showed six weeks of single-legged resistance exercise, performed with weight-loaded machines was also effective for improving insulin-stimulated blood flow during a

hyperinsulinemic-euglycemic clamp in healthy subjects and subjects with T2D. Short-term exercise interventions have also improved endothelial responsiveness during the postprandial state. Mikus *et al*. (79) showed that seven days of aerobic exercise improved postprandial leg blood flow in response to an oral glucose load in subjects with T2D (Figure 2.8). Furthermore, the improvement in postprandial leg blood flow occurred without any changes in metabolic outcomes (79).

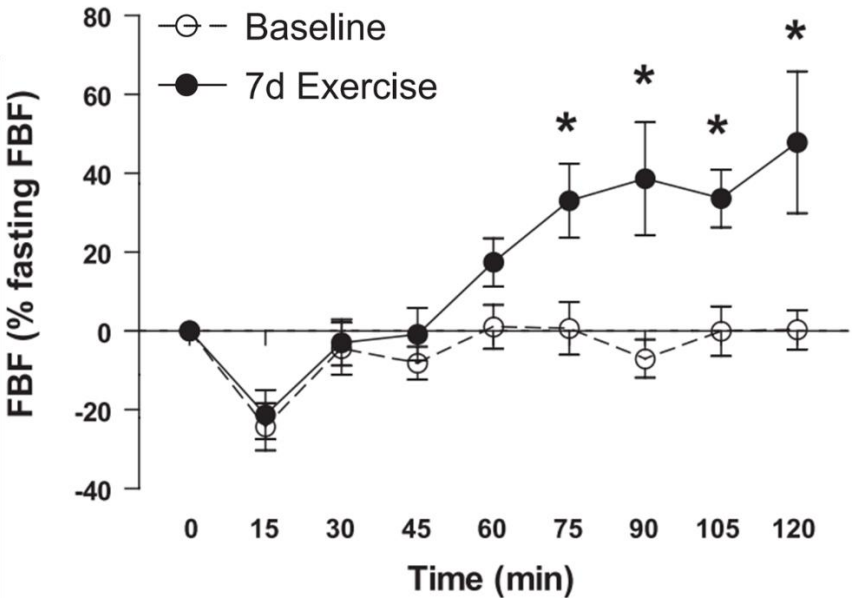

Figure 2.8 Seven days of aerobic exercise improved relative femoral blood flow (percent change from fasting) in response to 75g oral glucose load in subjects with T2D. *Significantly different from the corresponding time point at baseline ($P < 0.05$). FBF, femoral blood flow. Adapted from Mikus *et al.* (79).

Acute exercise has further demonstrated the effectiveness of exercise in improving endothelial insulin responsiveness. Bisquolo *et al.* (212) showed in healthy subjects that insulin-stimulated blood flow was greater during a hyperinsulinemic-euglycemic clamp 90-minutes after a 45-minute bicycle

exercise. Sjoberg *et al*. (23) showed insulin-stimulated microvascular perfusion of skeletal muscle was increased in the exercised leg compared to an unexercised contralateral leg four hours after single-legged exercise. Additionally, glucose uptake was increased in the exercise leg compared with the rested leg. Notably, the augmented effect of exercise on improving insulin-stimulated blood flow and perfusion and glucose uptake was abrogated when a NOS inhibitor was co-infused, indicating that the coordinated improvements between vasculature and skeletal muscle were NO-dependent.

Shear stress - a primary mechanism for exercise-mediated improvements in endothelial insulin resistance - is a physiological response shared by passive heating

A primary mechanism for exercise-mediated improvements in vascular dysfunction and endothelial responsiveness comes from exercise-induced elevations in blood flow and subsequently increased endothelial shear stress (145). Indeed, both exercise and passive heating mediated increases in endothelial shear stress have been demonstrated to be a key stimulus for inducing vascular adaptations (213, 214). A series of studies have demonstrated that shear stress is essential for arterial adaptation to exercise and passive heating (214-218). In these studies, measurements of vascular function were assessed in both arms before and after an intervention. During the intervention phase of these studies, one arm was occluded with a blood pressure cuff, preventing blood flow and shear stress from increasing above resting levels,

whereas the other arm was allowed to experience increased blood flow and shear stress during the intervention sessions. Subsequently, arterial adaptations in both the macro and microvasculature, as measured by increased brachial artery FMD responses and cutaneous microvascular endothelial function were prevented in the occluded arm following eight weeks of local arm heating (214), waist level hot water immersion (215, 216), lower limb exercise training (217), and handgrip exercise training (218).

Exposures to increased endothelial shear stress could improve endothelial insulin responsiveness through several mechanisms. Cell culture studies and isolated perfused arteries have demonstrated that increased shear stress increases eNOS expression (219, 220). Woodman *et al*. (219) found that isolated arteries perfused with higher shear rates over four hours induced greater eNOS expression and augmented acetylcholine-induced dilation. Therefore, increased eNOS expression following increased shear stress promotes greater responsiveness to endothelial-dependent dilators, much like insulin.

Cell culture studies have also shown that increased shear stress reduces ET-1 expression in a dose-dependent fashion (221). When a NOS inhibitor was applied, the shear-stress mediated reduction of ET-1 was negated. These data suggest that shear-mediated reduction of ET-1 is NO-dependent (232). The reduction of ET-1 is significant for two reasons. Firstly, reducing ET-1 can suppress proatherogenic signals and actions. Secondly, available NO can suppress ET-1 activity (146-149), therefore with less ET-1, there is increased

bioavailability of NO that could promote augmented dilatory responses or allow NO to participate in other antiatherogenic actions.

Notably, previous work has shown in cultured endothelial cells and isolated arteries that applying shear stress for one hour subsequently augmented insulin signaling and insulin-induced dilation, respectively (128). These observations were recapitulated in healthy humans, in which, after an hour of single-leg heating, insulin-stimulated blood flow and skeletal muscle microvascular recruitment and perfusion were augmented in the heated leg and not the control leg (128). Importantly, heating was used to increase blood flow and endothelial shear stress independent of contraction-induced increases in shear stress (128). Interestingly, lower body hot water immersion has been demonstrated to increase shear rate more than treadmill running (198), suggesting passive heating could be a more potent stimulus than exercise (Figure 2.9). Nevertheless, further studies are needed to confirm this.

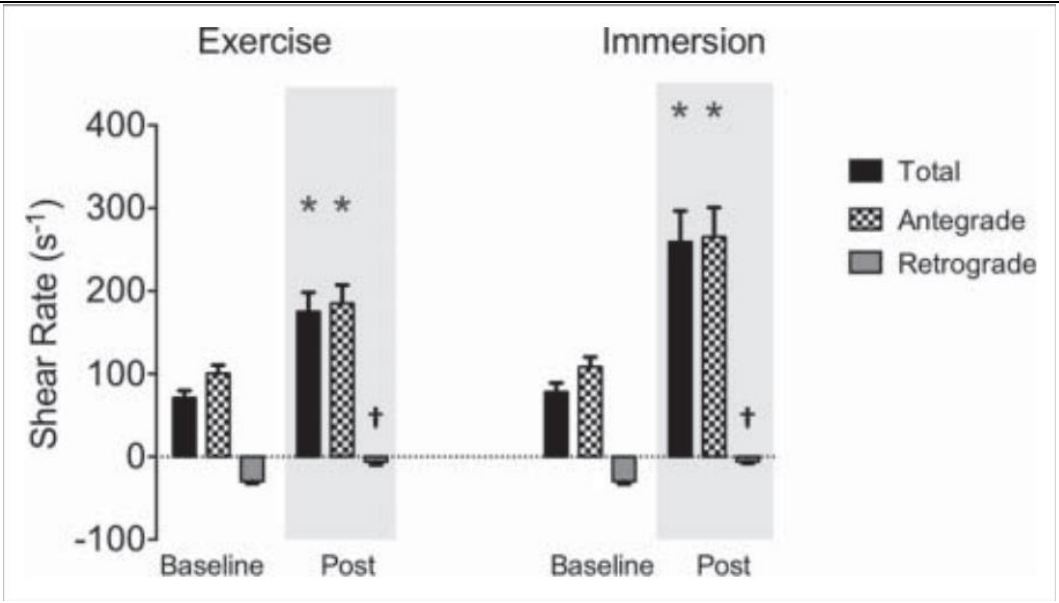

Figure 2.9 Superficial femoral artery shear rate increased more by hot water immersion than treadmill running. Young healthy subjects either participated in hot water immersion (30-minutes of waist level immersion in 42°C water) or treadmill running exercise (30-minutes 65-75% age-predicted heart rate maximum). * interaction: intervention x time ($p<0.05$); † different from baseline ($p<0.05$). Adapted from Thomas *et al.* (198).

Both exercise and passive heating increase endothelial shear stress that has been demonstrated to improve endothelial inulin sensitivity, while passive heating to improve endothelial insulin responsiveness in patients with T2D has not been investigated. It is reasonable to postulate that passive heating could improve endothelial responsiveness in patients with T2D via increasing endothelial shear stress and or independent of induced HSP72.

Passive heating could be an alternative therapy to exercise

In addition to passive heating and exercise sharing the ability to increase endothelial shear stress, passive heating induces other similar physiological

responses and adaptations as an exercise that promote health benefits (191, 222). A recent mini-review by Cullen *et al*. (223) has highlighted such similar adaptations which are illustrated in Figure 2.10.

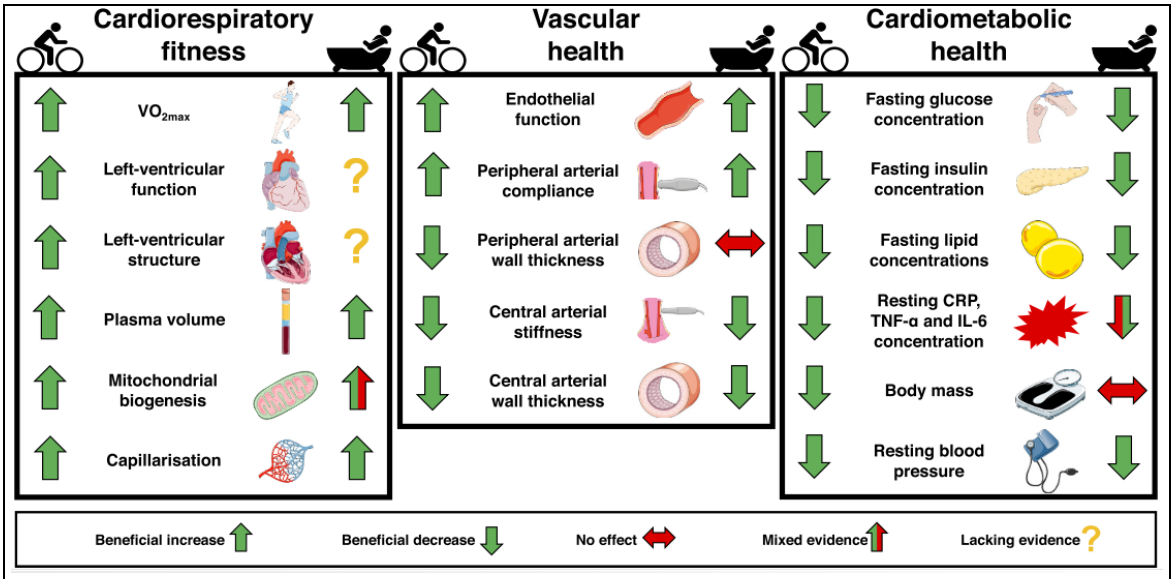

Figure 2.10 Summary of chronic adaptations to exercise and passive heating. Adapted from Cullen *et al.* (223).

Clinical studies directly comparing the effectiveness of exercise against passive heating are limited, yet, passive heating has been demonstrated to be as effective as exercise. Eight weeks of hot water immersion (42°C water, 30-minutes/session, 3 sessions/week) and time-matched moderate intensity, improved brachial artery FMD, cerebral blood flow, thermoregulatory function, and cardiorespiratory fitness to the same extent in young women (224). Six weeks of sauna-bathing induced similar improvements in postprandial glycemic control, cardiorespiratory fitness, and skeletal muscle microvascular adaptations such as capillarization and eNOS content, as time-matched moderate-intensity

cycling in young sedentary men (225), but, unlike exercise, sauna-bathing did not increase skeletal muscle mitochondrial density, GLUT 4 content, or intramuscular triglyceride content (225).

Notably, passive heating has been as effective as exercise in treating clinical populations. In PAD patients, twelve weeks of hot water immersion (39°C water, 30-minutes, 3-5 times/week) was as effective as aerobic and resistance exercise (90 minutes, 2-3 times/week) in improving tissue oxygenation during symptom-free walking, functional walking capacity, and blood pressure responses (117). However, the passive heating intervention was more effective than the aerobic and resistance exercise intervention at reducing systolic blood pressure in these patients with PAD (117). Similarly, Thomas *et al*. (198) found reduced blood pressure in young, healthy subjects after an acute session of waist level hot water immersion (42°C water, 30-minutes) yet not after treadmill running (65-75% HR max for 30 minutes).

The effectiveness of passive heating is clinically relevant as patients with T2D often have reduced cardiorespiratory fitness and a decreased capacity for sustained exercise (92-99). Furthermore, patients with T2D often cite barriers to exercise that relate to physical and mental discomfort that perpetuate unmotivated behaviors (100, 101). Therefore, passive heating could be an appealing treatment for individuals who cannot exercise, are discouraged by it, or choose not to participate. Collectively, passive heating could be a promising alternative therapy to exercise for patients with T2D.

SUMMARY & GAPS IN THE LITERATURE

The prevalence and incidence of T2D are growing (1, 2) due to obesity (4, 5) and physical inactivity (6, 7), known prime drivers of T2D (8). Individuals with T2D are at increased risk for CVD and CVD-related mortality (9, 10). Impaired endothelial insulin signaling of the PI3K-Akt pathway contributes to CVD and glycemic dysregulation, which characterize T2D and increases CVD risk (12, 16-18). As endothelial insulin resistance represents a causal factor in the pathogenesis of atherosclerosis and T2D, there is an urgency to identify the molecular mechanisms that cause it and subsequently devise effective treatments.

Mediators such as obesity, physical inactivity, glucolipotoxicity, and subsequent inflammation increase stress kinases such as JNK (23, 127), which disrupt the PI3K-Akt insulin signaling pathway (40-42). Therefore, targeting such stress kinases through regulatory mechanisms may restore endothelial insulin signaling. HSP72, a heat-inducible cytoprotective protein chaperone molecule, has been implicated in insulin resistance. Reduced HSP72 has been shown in metabolically active tissues from insulin-resistant humans (55-59) and animals (57, 62, 63) and in the aorta of insulin-resistant rats (64). The reduction may be due to suppressive actions by JNK (65-68). Increasing HSP72 can improve insulin sensitivity (57, 61, 63, 64, 69, 70, 72-76), insulin-stimulated PI3K insulin signaling (57, 61, 63, 70, 73, 76) and reduce JNK activity (57, 61, 63, 75, 76), likely due to its ability to mitigate JNK activity (44-47).

Based on the presented evidence, HSP72 may be involved with endothelial insulin resistance, yet this remains unknown. HSP72 is reduced in isolated aortas from insulin-resistant rats (64), and JNK activity is elevated in endothelial cells from patients with T2D (42) and vessels from individuals with severe obesity (43). Accordingly, it was hypothesized that endothelial insulin resistance in T2D is attributable to reduced endothelial HSP72. Conversely, increasing HSP72 may improve endothelial insulin responsiveness, perhaps by its inhibitory effects on JNK (44-47). It was hypothesized that increasing HSP72 would improve or augment endothelial insulin responsiveness.

In line with this, it is unknown if modulating HSP72 affects endothelial insulin signaling. Silencing HSP72 has been reported to impair VEGF-stimulated Akt that is needed for angiogenesis (51). Furthermore, increasing aortic HSP72 with passive heating improved angiotensin 1-7 stimulated Akt-eNOS activity resulting in improved vasodilation in insulin-resistant rats (64). HSP72 may be necessary for endothelial processes involving Akt signaling, including endothelial insulin signaling. It was hypothesized that decreasing HSP72 impairs endothelial insulin signaling, whereas increasing HSP72 would be beneficial.

Passive heating has emerged as a potential therapy for treating cardiometabolic diseases (108, 117, 123-125). It has been demonstrated that acute passive heating can improve endothelial insulin responsiveness in healthy subjects (128). This treatment could be leveraged to treat patients with T2D. Accordingly, it was hypothesized that passive heating would improve endothelial

insulin responsiveness in subjects with T2D and that this insulin-sensitizing effect of passive heat could be due to increasing endothelial HSP72.

Based on the presented gaps in the literature, this dissertation determined if endothelial insulin resistance in T2D is attributable to decreased endothelial HSP72. This dissertation also established if passive heating improves endothelial insulin responsiveness in subjects with T2D, in part by increasing endothelial HSP72. Furthermore, this dissertation determined if modulation of HSP72 impacts endothelial insulin signaling in cell culture, in that reducing HSP72 impairs endothelial insulin signaling, whereas upregulating HSP72 improves or augments it.

Beyond this dissertation and based on information within this literature review, targeting HSP72 may be beneficial for treating other vascular dysfunctions in T2D that include atherosclerosis (174, 175), hypertension (180), and impaired angiogenesis (51). Although heat-mediated increased HSP72 may be beneficial, it is not the only mechanism whereby heating may be beneficial for T2D. Notably, heat-mediated increases in endothelial shear stress may be a mechanism for the insulin-sensitizing effect of passive heating on the vasculature (128). Passive heating has been shown to improve vascular function (118, 122, 123, 197-199), glycemic control (124, 125), and reduce overactive sympathetic activity (123), all factors which would benefit individuals with T2D. However, more clinical studies are needed with T2D cohorts. While strategies such as exercise effectively treat T2D (226), exercise may be challenging for individuals with T2D (92-99), and passive heating may be a potential treatment alternative.

CHAPTER THREE – MAIN FINDINGS

Endothelial HSP72 is not reduced in type 2 diabetes nor is it a key determinant of endothelial insulin sensitivity

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Running Title: Role of HSP72 in endothelial insulin sensitivity

Key Words: Endothelial insulin resistance; insulin signaling; heat shock protein 72; leg blood flow; passive heating

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ABSTRACT

Impaired endothelial insulin signaling and consequent blunting of insulin-induced vasodilation is a feature of type 2 diabetes (T2D) and contributes to vascular disease and glycemic dysregulation. However, the molecular mechanisms underlying endothelial insulin resistance remain poorly known. Herein, the hypothesis that endothelial insulin resistance in T2D is attributed to reduced expression of HSP72 was tested. HSP72 is a cytoprotective chaperone protein that can be upregulated with heating and is reported to promote insulin sensitivity in metabolically active tissues, in part via inhibition of JNK activity. Accordingly, it was further hypothesized that, in T2D individuals, seven days of passive heat treatment via hot water immersion to waist-level (one hour/day) would improve leg blood flow responses to an oral glucose load (*i.e.*, endogenous insulin stimulation) via induction of endothelial HSP72. Contrary to the hypotheses, it was found that: 1) endothelial insulin resistance in T2D mice and humans was not associated with reduced HSP72 in aortas and endothelial cells, respectively; 2) after passive heat treatment, improved leg blood flow responses to an oral glucose load did not parallel with increased endothelial HSP72; 3) downregulation of HSP72 (via small-interfering RNA) or upregulation of HSP72 (via heating) in cultured endothelial cells did not impair or enhance insulin signaling (*i.e.*, activation of Akt), respectively, nor was JNK activity altered. Collectively, these findings do not support the hypothesis that reduced HSP72 is a key driver of endothelial insulin resistance in T2D but provide novel evidence

that lower-body heating may be an effective strategy for improving postprandial blood flow.

NEWS & NOTEWORTHY

Data from the present investigation using various experimental models including isolated mouse arteries, humans, and cultured endothelial cells suggest that endothelial insulin resistance in type 2 diabetes (T2D) is not causally attributed to reduced expression of heat shock protein 72. Furthermore, data from this study shows for the first time that lower-body heating may be a promising approach for restoring leg vascular insulin sensitivity in individuals with T2D.

INTRODUCTION

The prevalence and incidence of type 2 diabetes (T2D) are growing in the United States and worldwide (1, 2), with the number of US adults diagnosed with T2D expected to nearly triple by 2060 (3). Notably, individuals with T2D are at increased risk of developing and subsequently dying from cardiovascular disease (9, 10). Endothelial cell dysfunction plays an important role in the pathogenesis of cardiovascular disease in T2D (12-14, 16). Particularly, impaired endothelial insulin signaling through the PI3K-Akt pathway and consequent blunting of insulin-induced vasodilation and blood flow, also referred to as selective endothelial insulin resistance (14, 17), contributes to vascular disease and glycemic dysregulation (12, 16-18). Indeed, genetic disruption of insulin signaling in endothelial cells promotes atherosclerosis (21) and limits skeletal muscle glucose uptake (29), while conversely, selective activation of the insulin receptor-PI3K-Akt signaling pathway protects against atherosclerosis formation (130). Notwithstanding the unquestionable recognition that vascular insulin resistance is implicated in the pathogenesis of cardiovascular and metabolic diseases, the molecular underpinnings underlying endothelial insulin resistance in T2D remain poorly known. A deeper understanding of such molecular mechanisms can help identify therapeutic strategies for the prevention and treatment of T2D-associated vasculometabolic derangements.

Heat shock protein 72 (HSP72) is a heat-inducible cytoprotective chaperone protein whose constitutive expression appears to be suppressed in obesity and T2D, particularly in metabolically active tissues (56-58). Importantly,

deficiency of HSP72 promotes insulin resistance (57, 61) and these effects are likely mediated through activation of c-Jun amino-terminal kinase (JNK) (44-46, 57, 61, 76), a classic stress-activated kinase shown to disrupt the PI3K-Akt insulin-signaling pathway (40-42). However, the role of HSP72 in modulating insulin signaling in the vasculature remains unknown. Herein, it was hypothesized that endothelial insulin resistance in T2D can be attributed to reduced expression of HSP72.

Accumulating evidence indicate that chronic passive heating is therapeutically effective in treating and preventing cardiometabolic diseases (106-108, 117, 122-124, 126, 187, 190). Along these lines, a recent study showed that one bout of lower-limb heating subsequently increases insulin-stimulated leg blood flow in healthy individuals (128). It is possible that the multifaceted beneficial effects of passive heating, including its insulin-sensitizing actions, are at least in part driven by an induction of HSP72. As such, it can be reasoned that passive heating may be an effective strategy for restoring HSP72 expression in T2D and thus correcting endothelial insulin resistance. Specifically, it was hypothesized that, in individuals with T2D, passive heat treatment via hot water immersion would improve leg blood flow responses to an oral glucose load (*i.e.*, endogenous insulin stimulation), and that this improvement would be accompanied by an induction of HSP72 in endothelial cells. Congruently, it was also posited that knockdown of HSP72 in endothelial cells would impair insulin signaling and that the converse would also be true; that is, induction of HSP72 in endothelial cells would enhance insulin signaling.

METHODS

Experimental protocol in isolated arteries from mice

All animal procedures were approved by the University of Missouri Institutional Animal Care and Use Committee. The University of Missouri is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Isolated aortic rings were used from 20-week-old control db+ (n=12, 32.5±0.73 g body weight) and diabetic db/db (n=10, 58.1±1.02 g body weight) male mice (Strain #000642; The Jackson Laboratory, Bar Harbor, ME). Males were used because of availability. Aortas were harvested and cleaned of perivascular adipose tissue in ice-cold physiological saline solution (pH 7.4) and cut into 2-mm segments. Abdominal aortic rings were then mounted on wire myograph organ bath chambers (620M; Danish Myo Technology, Hinnerup, Denmark) containing warmed physiological saline solution gassed with 95% O_2-5% CO_2 and maintained at 37°C as previously described (227, 228). Aortic rings were treated with 80mM KCl to ensure viability. Next, aortas were preconstricted with the thromboxane A2-mimetic, U-46619 (20nM) to test vasorelaxation responses to increasing insulin concentrations (10^{-9} to 10^{-5}M, Humulin R; Eli Lilly, Indianapolis, IN). The remaining aorta was flash-frozen and subsequently processed for analysis of HSP72 and phospho-JNK via western blotting, as described below.

Experimental protocol in human subjects

Human participants

The study was approved by the University of Missouri Institutional Review Board (IRB, no. 2008181), registered at ClinicalTrials.gov (NCT03203694) and conducted in accordance with the Declaration of Helsinki. Subjects with a self-reported clinical diagnosis of non-insulin-dependent T2D, along with age- and sex-matched healthy subjects, were recruited from the Columbia, MO area. All subjects provided written informed consent and completed a medical health history questionnaire before participating in the study. All subjects were 35-65 years old and free of overt cardiovascular, renal, hepatic, autoimmune diseases, cancers, exogenous insulin use, immunosuppressant therapies, gout, diabetic neuropathy, tobacco or nicotine use, excessive alcohol consumption (>14 drinks per week for men, and >7 drinks per week for women), pregnancy or nursing, and mobility limitations. Furthermore, subjects with T2D were also excluded if they had a body mass index (BMI) of ≥50 kg/m^2, self-reported participating in more than 60 minutes of exercise per week, had uncontrolled hypertension (≥180mmHg systolic, or ≥110mmHg diastolic), or had any fungal infections or disorders. Additionally, based on manufacturer contraindications for the sensor used for measuring core temperature during the heating sessions, subjects were excluded if they presented with any of the following: swallowing or esophageal disorders, gag-reflex impairment, gastrointestinal tract diseases or disorders, any previous gastrointestinal surgeries, any implanted electromedical device, or any scheduled nuclear magnetic resonance/magnetic resonance imaging scanning unrelated to the study. Healthy subjects were also excluded if they had a BMI outside of the 19-29 kg/m^2 range, a history of pre-diabetes or diabetes mellitus,

self-reported participating in <150 minutes of moderate-intensity exercise per week, had hypertension (≥130mmHg systolic, or ≥90mmHg diastolic) or were taking any anti-hypertensive medications. The use of non-obese, physically active adults as healthy control subjects allowed us to characterize the optimal vascular and metabolic phenotype to be used for reference. This idea that control subjects should be physically active has been persuasively advocated by Booth and colleagues (229-233). Five women (n=2 from T2D group, n=3 from healthy group) were premenopausal and had their experimental visits scheduled during the early follicular phase (days 1 – 7 of the menstrual cycle) or oral contraceptive placebo week (if applicable) to minimize any potential impact of hormonal fluctuations across the menstrual or oral contraceptive cycle on metabolic and vascular outcomes. Therefore, ~28 days elapsed between experimental visits for these participants.

Experimental design

Healthy subjects participated in a single experimental visit. Subjects with T2D participated in two experimental visits, one occurring before and the other after seven consecutive days of passive heating sessions. The second experimental visit occurred 16-24 hours after their last passive heating session, as previously described in other passive heating studies (207). Such short-term course of treatment (*i.e.*, seven days) was purposely used to examine the vascular effects of heating prior to overt improvements in metabolic function, known to occur with longer heating interventions (125-127), which could in turn

lead to secondary vascular effects. That same strategy has been employed in exercise studies with individuals with T2D using similar outcomes (79).

The seven-day lead up to experimental visits

Prior to experimental visits, subjects wore an accelerometer for seven consecutive days. Subjects with T2D also wore an accelerometer during the seven consecutive days of passive heating sessions prior to their second experimental visit. All subjects filled out a three-day food diary prior to an experimental visit. Subjects with T2D were given a copy of their food intake record and instructed to replicate the meals and timing of food intake, as much as possible, for the three days before their second experimental visit.

Experimental visit

The experimental visit and measurements taken are illustrated in **Appendix I Figure 1**. Participants arrived at the laboratory after an overnight fast. Subjects refrained from medications the morning of testing and also abstained from exercise for 24-48 hours, caffeine for 12 hours, and alcohol for 24 hours before testing. Subjects underwent anthropometric measurements including height, weight, and body composition via dual-energy X-ray absorptiometry (HorizonA, Hologic Inc., Bedford, MA). After 15 minutes of supine rest in a darkened, temperature-controlled room (~21°C), aortic stiffness via carotid-to-femoral pulse wave velocity (cfPWV) and femoral artery endothelial function via flow-mediated dilation (FMD) were assessed to further characterize

vascular function. An intravenous (IV) catheter was then placed in an antecubital vein for blood sampling and collection of venous endothelial cells. Thereafter subjects underwent an oral glucose tolerance test to assess leg blood flow responses to endogenous insulin stimulation (*i.e.*, a physiological readout of vascular insulin sensitivity).

Experimental measurements

Physical activity

Subjects were fitted with an accelerometer (ActiGraph GTX3; ActiGraph, Pensacola, FL) on the right hip to record physical activity. Accelerometers collected data at a rate of 30Hz for seven consecutive days. Subjects were instructed to wear the accelerometer upon waking until just prior to sleeping, and were given a diary to note what periods they wore the accelerometer and any reason for taking the device off (*e.g.*, sleeping, bathing, etc.).

Recorded data were downloaded over 60-second epochs and analyzed with the manufacturer's software (ActiLife v6.13.3, ActiGraph, Pensacola, FL). ActiLife wear-time validation was performed and, subsequently, the amount of time spent in moderate-vigorous physical activity per week was calculated. Moderate-vigorous physical activity was determined by the ActiLife software using device counts per minute and cut-off thresholds for adults published by Freedson (234). Time spent in moderate-vigorous physical activity per week was selected as the reference for physical activity based upon American national guidelines for promoting health benefits (235).

Aortic stiffness via cfPWV

cfPWV was measured using the cuff-based SphygmoCor® XCEL (AtCor Medical, Itasca, IL) to assess aortic stiffness, according to current recommendations and as previously described (236, 237). Briefly, the SphygmoCor® XCEL device enables simultaneous acquisition of carotid (via tonometer) and femoral (via cuff) pulse waves. Transit time between carotid and femoral pressure waves was calculated using the foot-to-foot method. Wave foots were identified using intersecting tangent algorithms. cfPWV (reported in m/s) was calculated as distance traveled by the pulse wave divided by pulse transit time. For nine T2D subjects, the carotid pulse wave signal was of insufficient quality and thus cfPWV data could only be generated for a subset of the subjects.

Femoral artery endothelial function via FMD

FMD in the superficial femoral artery was performed via 2D/Doppler ultrasound (GE Logiq P5) according to published guidelines (238) and as previously described (239-241). Two minutes of arterial diameter and velocity were recorded using an 11MHz linear array transducer. Signals were obtained in duplex mode at a pulsed frequency of 5MHz and corrected with an insonation angle of 60 degrees. Sample volume was adjusted to encompass the entire lumen of the vessel without extending beyond the walls, and the cursor was set at mid-vessel and parallel to the vessel wall. A cuff placed on the calf muscle was then inflated to a pressure of 250mmHg for five minutes. Continuous

diameter and blood velocity measures were recorded during this occlusion period and three minutes following cuff deflation. For subjects with T2D, the ultrasound probe placement was marked on the skin, markings were scaled against a tape measure, and a picture was taken to ensure consistency between visits.

Video recordings of the ultrasound images were obtained with real-time capture software (Elgato Video Capture, Elgato, CA, USA), later exported and analyzed using an automated wall detection software (Cardiovascular Suites 4, Quipu Srl, Pisa, Italy). FMD percent change was calculated as [(peak diameter-base diameter)/base diameter] x 100. Shear rate, an estimate of shear stress without blood viscosity, was calculated as 4 x mean blood velocity/diameter. Post-occlusion hyperemic shear rate area under the curve (AUC) up to 60 seconds was also calculated (33, 242).

Venous endothelial cell collection and processing

After creating a sterile field, a 20-gauge IV catheter was placed in an antecubital vein using aseptic techniques. A 0.18” spring-wire J-tip guidewire (AW-16402, Arrow International, Reading, PA) was advanced ~2-5 inches through the catheter, moved back and forth 10 times to collect endothelial cells. The wire was cut and placed into a conical tube containing a disassociation buffer. This process was repeated for a total of four guidewires.

The collected wires were rinsed with dissociation buffer for 10 minutes using a motorized serological pipette. Red blood cell lysis buffer (eBioscience 1X RBC Lysis Buffer #00-4333-57, Invitrogen, Thermo Fisher Scientific, Carlsbad,

CA) was added to the collected cells for 10 minutes at room temperature. Cells were then centrifuged, washed, and subsequently fixed in pellet form with 4% paraformaldehyde (PFA, #15710, Electron Microscopy Sciences, PA) for 10 minutes at room temperature. Fixed cells were washed, centrifuged, resuspended, and 10μL of fixed cell suspension was plated per well onto a 15-well chambered microscope slide (μ-Slide Angiogenesis ibiTreat #81506, Ibidi GmbH, Gräfelfing, Germany) pre-treated with poly-L-lysine solution (P4832, Sigma Aldrich, St. Louis, MO). Slides were then incubated at 37°C for five hours to allow cells-to-slide adherence and then rinsed with 50mM glycine/PBS solution to quench any remaining PFA. Slides were stored at -80°C until further analysis of proteins of interest via quantitative immunofluorescence staining.

Quantitative immunofluorescence staining of venous endothelial cells

Slides were thawed and rehydrated with PBS for 10 minutes. Slides were then incubated with permeabilization buffer (0.05% Triton X-100 + 2% BSA + PBS) and then blocking buffer (Goat serum + 0.05% Triton X-100 + 1% BSA + PBS) for one hour each at room temperature. Slides were incubated overnight at 4°C with primary antibodies for the following targets: von Willebrand factor (vWF) (1:1000; Invitrogen, # PA1-43057), phospho-JNK (Thr183/Tyr185 (1:100; Cell signaling #9255), JNK (1:100; Cell Signaling, #9252), and HSP72 (1:50; Enzo Life Sciences, #ADI-SPA-810). Slides were incubated the following day with corresponding fluorophore-conjugated secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI) (1:500 or 1:1000; Sigma Aldrich, #D9542) for one hour at

room temperature. The fluorophore-conjugated secondary antibodies used were Alexa Fluro 488 (1:2000; Abcam #150177), Alexa Fluro Plus 555 (1:250; Invitrogen # A32727; or 1:500; A32732), and Alexa Fluro 633 (1:500; Invitrogen # A21071).

Each multi-well chambered slide accommodated the simultaneous probing of phospho-JNK, JNK, and HSP72. Phospho-JNK and JNK were probed for within the same wells. All wells were probed for DAPI and vWF. DAPI identified intact cell nuclei while vWF was used to identify endothelial cells with intact cell membranes. Staining of slides was performed in several batches. Each batch contained four slides: one slide from a healthy subject, two slides from a T2D subject (before and after their heating intervention), and one slide containing human vein endothelial cells (#CC-2159, Lonza, Walkersville, MD) at passage four used for normalization (243, 244). Slides were imaged on an 63x oil objective (1.4 numerical aperture) using the Leica Thunder Imager (Leica Microsystems, Wetzlar, Germany). Images were captured at the same exposure time and corrected for background fluorescence using Leica Thunder software. The mean fluorescence intensity per unit area of the proteins of interest was calculated from 27±2 cells per subject and normalized to the respective mean fluorescence intensity from HUVECs (simultaneously stained) to account for any batch effect. The fluorescence intensity was analyzed using a custom MATLAB script that automated the identification of positively stained endothelial cells (*i.e.*, only signal from endothelial cells was used for analysis). Cells with a compromised membrane and considered not spherical by the script's algorithm

were excluded from the analysis. The imaging and fluorescence intensity analysis was completed by an investigator blinded to the characteristics of subjects and treatments.

Leg blood flow responses to glucose ingestion

After a 40-minute period of supine rest, a 75-g oral glucose load (#100075, Azer Scientific, Morgantown, PA) was ingested within five minutes. Before and during the postprandial state (15, 30, 45, 60, 90, and 120 minutes after ingestion), blood samples were collected from the IV catheter, and superficial femoral artery blood flow was assessed via 2D/Doppler ultrasound. Each blood flow recording period was at least four minutes long. At these time points, brachial artery blood pressure readings were also collected in duplicate using an automated blood pressure monitor (SunTech Tango M2, SunTech Medical, Morrisville, NC). Blood flow (reported in mL/min) was calculated as: 3.14 x (diameter (cm)/2)^2 x mean blood velocity (cm/s) x 60. Mean arterial pressure (MAP) (reported in mmHg) was calculated as: (2 x diastolic pressure) + systolic pressure)/3. Throughout, blood samples were placed in EDTA vacutainers and immediately analyzed for glucose using the YSI 2300 STAT PLUS glucose analyzer (YSI Inc., Yellow Springs, Ohio) or frozen for later determination of glycosylated hemoglobin (HbA1c) at the University of Missouri Diabetes Diagnostic Lab. The remaining blood samples were centrifuged (4°C, 3500 rpm, for 15 minutes), and the plasma was aliquoted and stored at -80°C for later analysis of insulin levels using a commercially available enzyme-linked

immunosorbent assay (#80-INSHU-E10.1, ALPCO, Salem, NH). Postprandial glucose and insulin incremental AUC were calculated with the trapezoidal method over the two-hour period. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as: (fasting glucose (mg/dL) x fasting insulin (μU/mL)) / 405.

Passive heating sessions

The passive heating sessions and measurements taken are illustrated in **Appendix I Figure 2**. Subjects were instructed to maintain their usual physical activity, dietary habits, and medication intake during the seven days of the heating intervention. At least 7-9 hours before arriving for their first and last session, the subject ingested a telemetry sensor (CorTemp Temperature Sensor HT150002, HQ Inc., Palmetto, FL) to measure core temperature (reported in °C). This sensor emits radio frequencies picked up by the receiver (HQinc, Palmetto, FL), placed near the lumbosacral region. Prior to each heating session, subjects provided a urine sample to assess hydration status using urine specific gravity (USG) readings from a digital refractometer (PAL-10S, Atago Co. LTD., Japan). If the USG reading was >1.02, indicative of dehydration, the subject drank 500mL of water. After five minutes of rest in a semi-recumbent chair, tympanic membrane temperature (TMT) was assessed with an infrared digital thermometer (Braun ThermoScan 5 IRT6500, Kaz USA Inc., MA) and brachial artery blood pressure was assessed with an automated blood pressure monitor (SunTech Tango M2). After recording nude body weight using a digital scale (Patient Aid

Scale PA-550XL, Patient Aid LLC, OH), subjects were also fitted with a heart rate monitor (Polar H10, Polar Electro Oy, Finland). Nude body weight and TMT were recorded for safety monitoring purposes only, hence these data are not reported.

Subjects were immersed at waist level in 40.5°C water for 60 minutes in an inflatable hot tub (GoRelax inflatable hot tub PH050013, Shanghai Sunshine Development Co. LTD., Shanghai, China), every day for seven days. This modality of passive heating has been shown to increase core temperature (216), a stimulus known to upregulate HSP72 expression (80). Every five minutes during immersion, core temperature (on first and last session), TMT, rating of perceived exertion (RPE) using the Borg's 6-20 point scale (245), thermal sensation using the 13-point McGinnis categorical scale (246), and signs/symptoms were recorded. Heart rate and blood pressure were recorded every 10 minutes. For comfort, subjects were allowed to rest their arms on the sides of the hot tub and out of the water. Also for comfort, a box fan continuously blew over them. During immersion, subjects were given a 500mL bottle of water to drink ad libitum.

After the 60-minute water immersion, subjects sat in a semi-recumbent chair for a 10-minute cool-down period, during which the fan continued to blow over them. Signs and symptoms, vitals, and TMT remained monitored during the cool-down. Next, subjects recorded a post-immersion nude body weight. If there was a 1% loss in nude body weight between pre and post immersion, subjects consumed 500mL of water before leaving the facility. To document any heat acclimation, mean whole-body sweat rate (reported as L/h) for each session was

calculated by taking the difference in dry nude body weight between pre and post immersion and correcting for water intake, as previously described (122, 125).

Experimental protocol in human skeletal muscle microvascular endothelial cells (hSMMECs) in culture

hSMMECs (#H-6220; Cell Biologics; Chicago, IL) were cultured in complete VascuLife® EnGS medium with 10% FBS. Cells were maintained in a humidified incubator at 37°C and 5% CO_2 unless otherwise stated. Experiments occurred when cells were at passage four on 60mm dishes and ~90% confluency unless otherwise stated.

Small-interfering-RNA (siRNA) knockdown of HSP72 and insulin stimulation

hSMMECs were passaged onto 6-well plates and maintained in VascuLife® EnGS medium with 10% FBS containing growth factors and no antibiotics. Twenty-four hours after plating, cells were switched to VascuLife® EnGS medium with 1% FBS containing growth factors and no antibiotics. At ~70-80% confluency, cells were transfected for six hours with siRNA targeting HSP72 (20nM; #sc-29352, Santa-Cruz Biotechnology, Santa-Cruz, CA) or siRNA-scramble (20nM; siRNA-control A #sc-37007, Santa-Cruz Biotechnology) with Lipofectamine™ RNAiMAX transfection reagent (#13778150, Invitrogen, Life Technologies Corp, Carlsbad, CA) complexed in Opti-MEM™ reduced serum medium (#11058021, Gibco, Grand Island, NY). After the transfection period, media was replaced with VascuLife® EnGS medium with 1% FBS containing

growth factors and no antibiotics. Cells recovered for 48 hours, and this transfection protocol and recovery were repeated. After the second recovery period, cells were incubated with vs. without insulin (100nM; Humulin R; Eli Lilly, Indianapolis, IN) for 30 minutes and collected.

Heating to overexpress HSP72 and insulin stimulation

Twenty-four hours prior to initiation of the experiment, cells were serum-starved in complete VascuLife® EnGS medium with 0.5% FBS. Cells were then placed in a water-jacketed incubator set to 40.5°C and 5% CO_2, or kept at 37°C and 5% CO_2 (sham-treated), for 60 minutes, over five consecutive days. Treatments co-occurred in two adjacent incubators. After the 60-minute heat exposure, cells were placed back in the 37°C incubator (with the sham-treated cells) and allowed to recover. After a 60-minute recovery period, cell culture media for all dishes was refreshed. Twenty-four hours after the last heat/sham treatment (day 5), cells were incubated with vs. without 100nM insulin for 30 minutes and collected.

Exposure to a glucolipotoxic milieu and insulin stimulation

Twenty-four hours prior to initiation of the experiment, cells were serum-starved in complete VascuLife® EnGS medium with 0.5% FBS. Cells were then treated with a glucolipotoxic milieu or osmotic vehicle solution for 24 hours and subsequently incubated with vs. without 100nM insulin for 30 minutes and collected. The glucolipotoxic milieu was used as an approach to suppress

HSP72 and overall Akt activity. The glucolipotoxic milieu contained complete VascuLife® EnGS medium with 0.5% FBS, 30mM D-glucose (24mM added to the 6mM present in the basal medium), and 0.1mM palmitic acid-BSA conjugate (1mM:0.25mM; 4:1 molar ratio complex). The osmotic vehicle solution contained complete VascuLife® EnGS medium with 0.5% FBS, 24mM D-mannitol, and 0.1mM BSA-vehicle (0.25mM BSA).

Heating to restore expression of HSP72 in cells exposed to the glucolipotoxic milieu and insulin stimulation

Twenty-four hours prior to initiation of the experiment, cell culture media was switched to media containing the glucolipotoxic milieu. Cells were then placed in a water-jacketed incubator set to 40.5°C and 5% CO_2, or kept at 37°C and 5% CO_2 (sham-treated), for 60 minutes. Treatments co-occurred in two adjacent incubators. After the 60-minute heat exposure, cells were placed back in the 37°C incubator (with the sham-treated cells) and allowed to recover. After a 60-minute recovery period, the media containing the glucolipotoxic milieu was refreshed. Twenty-four hours after the heat/sham treatment, cells were incubated with vs. without 100nM insulin for 30 minutes and collected. Only a single bout of heating was used for this experiment, rather than the five-day paradigm, because exposure to the glucolipotoxic milieu for five days compromised cell viability (data not shown).

Cell lysate collection after insulin stimulation

Immediately following the 30-minute insulin stimulation, cells were washed with cold, sterile PBS and lysed in RIPA buffer (R0278, Millipore Sigma, St. Louis, MO) supplemented with EDTA and protease and phosphatase inhibitors. Cell lysates were collected via cell scraping and were subsequently sonicated and centrifuged. The supernatant was collected after the centrifugation and stored at -80°C for subsequent western blotting.

Western blotting

Cell lysates were prepared in 2x Laemmli buffer (#1610737, Bio-Rad Laboratories Inc, USA) following protein quantification using Pierce BCA protein assay. Prepared protein samples (3–5 µg/lane) were separated in Criterion™ Tris-Glycine-eXtended Stain-Free™ precast gels (#5678085, Bio-Rad Laboratories Inc, USA). Proteins were transferred overnight onto polyvinylidene difluoride membranes and blocked with 5% BSA for one hour at room temperature. Membranes were probed overnight at 4°C for: phospho-Akt (Ser473) (1:1000; Cell Signaling, #4060), Akt (1:1000; Cell Signaling, #9272), HSP72 (1:1000; Enzo Life Sciences, #ADI-SPA-810), phospho-JNK (T183/Y185) (1:1000; Cell Signaling #9251), JNK (1:1000; Cell Signaling, #9252), and GAPDH (1:1000; Cell Signaling, #5174). Secondary antibodies were applied the following day for one hour at room temperature, which included: anti-rabbit (1:2000; Biorad #1705046) and anti-mouse (1:2000; Cell Signaling, #7076). Blots were imaged (Bio-Rad ChemiDoc XRS+ System, Bio-Rad, Hercules, CA). The intensity of individual protein bands was quantified via densitometry using Image Lab

Software (v.6.0.1 build 35, Bio-Rad, Laboratories, Inc.). Proteins of interest were normalized to GAPDH expression. Values are expressed as fold differences.

Statistical analyses

GraphPad Prism (v9.3.0, GraphPad Software LLC, La Jolla, CA) was used for statistical analysis. Statistical comparisons were performed by two-tailed *t*-test, or multivariate analysis of variance (ANOVA), as appropriate, followed by Bonferroni adjusted pair-wise comparisons when significant interactions were found. No sex differences were detected on outcome variables, therefore, data from male and female subjects were pooled for analysis. Individual responses and mean ± standard error of the mean (SEM) are presented, as appropriate. For all statistical tests, significance was accepted at $P<0.05$.

RESULTS

Endothelial insulin resistance in T2D mice and humans is not associated with reduced HSP72 in aortas and endothelial cells, respectively.

As displayed in **Figure 3.1A**, using wire myography, aortic rings from diabetic db/db mice exhibited suppressed insulin-induced relaxation relative to aortic rings from control db+ mice (interaction $P=0.002$). Impaired insulin-induced relaxation in aortic rings from db/db mice was not accompanied with a reduction in HSP72 content ($P>0.05$, **Figure 3.1B**) nor with an elevation in phospho-JNK ($P>0.05$, **Figure 3.1C**) in aortic tissues.

Similarly, as shown in **Figure 3.1E,** individuals with T2D exhibited a blunted leg blood flow response (interaction *P*=0.005) to an oral glucose load and concomitant acute hyperinsulinemia compared to healthy subjects, notably between 30 and 45 minutes after glucose ingestion. Blood flow responses beyond 60 minutes after ingestion were not different between groups (*P*>0.05, data not shown). Impaired blood flow responses to the oral glucose load in T2D, indicative of vascular insulin resistance, was not paralleled with a reduction in HSP72 content (*P*>0.05, **Figure 3.1F**) nor with an elevation in phospho-JNK (*P*>0.05, **Figure 3.1G**) in endothelial cells. Demographic information and subject characteristics are summarized in **Table 3.1**. As expected, relative to healthy controls, T2D individuals also exhibited increased aortic stiffness as assessed via cfPWV (*P*<0.001, **Table 3.1**) and impaired endothelial function in the femoral artery as assessed via FMD (*P*<0.001, **Table 3.2**). Additionally, relative to healthy controls, individuals with T2D had higher postprandial blood glucose concentrations from 60 minutes and beyond (interaction *P*<0.001, **Appendix I Figure 3A**). Postprandial plasma insulin responses similarly increased for both groups (time effect *P*<0.001, **Appendix I Figure 3B**).

Improved leg blood flow responses to an oral glucose load after passive heat treatment does not parallel with increased HSP72 in endothelial cells.

Passive heating via water immersion in individuals with T2D (illustrated in **Figure 3.2A**) effectively increased core temperature over the course of the 60-minute heating period (time effect *P*<0.001, **Figure 3.2B**). Passive heating also

increased heart rate (time effect $P<0.001$, **Figure 3.2C**) and reduced MAP (time effect $P<0.001$, **Figure 3.2D**). It should be noted that the fall in blood pressure during passive heating was further accentuated at the seventh session of heating (interaction $P=0.004$, **Figure 3.2D**). The passive heating intervention was well-tolerated as during immersion subjects registered thermal sensation as "comfortable" (**Appendix I Figure 4A**) and RPE as "extremely light" (**Appendix I Figure 4B**); perceptions that did not change over the course of the seven-day intervention. Furthermore, whole-body sweat rates during immersion, while tending to increase, were not significantly different between the first and seventh heating sessions (1^{st} session: 0.468 ± 0.04 L/h; 7^{th} session: 0.610 ± 0.08 L/h, $P=0.09$), suggesting subjects only became partially heat acclimated to the intervention.

Importantly, as shown in **Figure 3.2E**, the heating intervention in T2D individuals improved leg blood flow responses (treatment effect $P=0.03$) to the oral glucose load and the concomitant acute hyperinsulinemia. This improvement in postprandial blood flow after seven days of heating occurred despite no evidence of increased HSP72 expression in endothelial cells ($P>0.05$, **Figure 3.2F**). Phospho-JNK was also not affected by the heating intervention ($P>0.05$, **Figure 3.2G**). As expected by design, this short-term heating intervention was of insufficient duration to alter metabolic outcomes. It was also insufficient to increase FMD or reduce cfPWV, although a trend was observed for the latter ($P=0.07$). These data and other phenotypic parameters are summarized in **Tables 3.3 and 3.4**. Furthermore, the postprandial blood glucose

and plasma insulin responses were unaltered by the heating intervention (time effect *P*<0.001, **Appendix I Figure 5A & 5B, respectively**)

Downregulation or upregulation of HSP72 in cultured endothelial cells does not impair or enhance insulin signaling, respectively.

As shown in **Figure 3.3**, siRNA-mediated knockdown of HSP72 (*P*=0.002, **Figure 3.3A**) did not enhance phospho-JNK (*P*>0.05, **Figure 3.3B**) and did not blunt insulin-induced activation of Akt (interaction *P*>0.05, **Figure 3.3C**) in cultured endothelial cells. Similarly, HSP72 induction after five consecutive days of heating (*P*<0.001, **Figure 3.3E**) did not suppress phospho-JNK (*P*>0.05, **Figure 3.3F**) and did not enhance insulin-induced activation of Akt (interaction *P*>0.05, **Figure 3.3G**).

Furthermore, exposure of endothelial cells to a glucolipotoxic milieu reduced HSP72 (*P*<0.001, **Figure 3.3I**) but did not enhance phospho-JNK (*P*>0.05, **Figure 3.3J**). While downregulation of HSP72 caused by glucolipotoxicity was associated with an overall suppression of phospho-Akt (*i.e.*, main effect of glucolipotoxic treatment, *P*<0.001), it did not impair activation of Akt in response to insulin (*i.e.*, interaction *P*>0.05, **Figure 3.3K**). Similarly, restoration of HSP72 via heating in glucolipotoxic cells (*P*<0.001, **Figure 3.3M**) did not reduce phospho-JNK (*P*>0.05, **Figure 3.3N**) and did not elicit an improvement in insulin-induced activation of Akt (interaction *P*>0.05, **Figure 3.3O**).

DISCUSSION

The primary findings of this investigation are three-fold. First, endothelial insulin resistance in T2D mice and humans was not associated with reduced HSP72 in aortas and endothelial cells, respectively. Second, after passive heat treatment in individuals with T2D, improved leg blood flow responses to an oral glucose load did not parallel with increased endothelial HSP72. Third, downregulation or upregulation of HSP72 in cultured endothelial cells did not impair or enhance insulin signaling, respectively, nor was JNK activity altered. In aggregate, these findings do not support the notion that reduced HSP72 is a key determinant of endothelial insulin resistance in T2D but do demonstrate that lower-body heating may be efficacious for improving postprandial blood flow in the leg vasculature, potentially by another mechanism.

The finding that mouse diabetic arteries with impaired insulin-induced relaxation did not display evidence of suppressed HSP72 expression suggest that loss of HSP72 cannot be an obligatory mechanism causing insulin resistance in vascular tissue. This disconnect between HSP72 expression and vascular insulin sensitivity is corroborated in the cohort of individuals with T2D who showed evidence of blunted leg blood flow responses to endogenous insulin stimulation (using an oral glucose load) despite no decrease in endothelial expression of HSP72. In further support of this disassociation, it was found that seven days of passive heating in T2D individuals improved leg blood flow responses to insulin stimulation but this improvement was not accompanied with an induction of HSP72, suggesting the idea that upregulation of HSP72 with

heating may not be a required mechanism to promote vascular insulin sensitivity. Additional evidence supporting the lack of a causal relationship between HSP72 and endothelial insulin sensitivity is revealed by the endothelial cell culture loss- and gain-of-function experiments. That is, the experiments show that knockdown of HSP72 via siRNA does not impair endothelial insulin signaling (*i.e.*, activation of Akt in response to insulin stimulation) and that the converse is also true. Induction of HSP72 via heating, in naïve cells or cells exposed to a glucolipotoxic milieu, does not enhance insulin-induced Akt activity.

This "uncoupling" between HSP72 and endothelial insulin signaling could be explained by the fact that, contrary to the hypothesis, modulation of HSP72 did not influence JNK activity, a known stress-activated kinase shown to disrupt the PI3K-Akt insulin-signaling pathway (40-42). The finding that alteration of HSP72 in endothelial cells did not influence JNK activity suggests the link between HSP72 and JNK, supported by other studies in metabolically active cells and tissues (44-47, 56-58, 61, 63, 75, 76), may not extrapolate to the endothelium. Notably, while these data do not support a role of HSP72 in regulating endothelial insulin sensitivity, this is not to infer that endothelial HSP72 is not implicated in other important physiological processes. In this regard, Shiota *et al*. (51) found that siRNA-mediated knockdown of HSP72 in endothelial cells impairs VEGF-stimulated PI3K-Akt signaling and angiogenesis.

While it was hypothesized that repeated bouts of lower-body heating, and consequent increases in core temperature, would be a sufficient stimulus for upregulation of HSP72 in endothelial cells (80), there is also some precedence in

the literature for heating interventions in humans to not result in an induction of HSP72 (*e.g.*, in biopsied skeletal muscle (127) and adipose tissue (125), or collected immune cells (126)). This is in contrast to heating studies in animals which typically demonstrate an induction of HSP72, likely attributable to exposure to higher temperatures (57, 61, 63, 70, 72-75). Of note, previous studies have shown that exercise-induced (71) and heat-induced (62) HSP72 expression in metabolically active tissues is blunted in rat models of insulin resistance. In this context, it is plausible that T2D is associated with a deficiency in the heat stress response (48, 49) and that this may have contributed to the lack of upregulation of HSP72 with heating. However, additional studies are needed to test this hypothesis.

Importantly, despite the lack of induction of HSP72 after seven days of passive heating in T2D individuals, there was an observed improvement in leg blood flow responses to an oral glucose load. This improvement is reminiscent of findings by Mikus *et al*. (79) where seven days of aerobic exercise was shown to enhance leg blood flow responses to an oral glucose load in T2D subjects. Because both lower-body heating and exercise increase leg blood flow and shear stress, it is reasonable to speculate that increased shear stress may be the shared mechanism whereby these two interventions promote insulin-sensitizing effects in the vasculature. In support of this proposition, it was recently shown in cultured endothelial cells and in isolated arteries that application of shear stress for one hour subsequently augmented insulin signaling and insulin-induced dilation, respectively (128). The observation that heating and exercise produce

analogous insulin-sensitizing effects in the vasculature is even true after a single, one-hour bout of unilateral leg heating (128) and unilateral leg exercise (23) in young healthy subjects, again reinforcing the idea that increased shear stress may be the principal driving stimulus mediating these beneficial vascular effects.

The notion that heating can recapitulate some of the beneficial vascular adaptations to exercise in human limbs is clinically relevant (191), particularly when considering that a significant fraction of T2D patients are incapable of performing sustained aerobic exercise (95-99) or would choose not to do so (100, 101). Notably, these findings extend those from previous studies indicating that lower body heating also prevents inactivity-induced leg vascular dysfunction in healthy subjects (202) and, when applied for eight weeks, it improves several indices of vascular function in overweight sedentary individuals (122) and in women who are obese with polycystic ovary syndrome (123). Further underscoring the clinical significance of heat therapy, data from a large prospective cohort study revealed that increased frequency of sauna bathing is associated with a reduced risk of fatal cardiovascular diseases and all-cause mortality (106). Furthermore, heat therapy has been demonstrated to improve the prognosis of patients with chronic heart-failure (108), and both vascular outcomes and physical functioning in patients with coronary artery disease (116), chronic heart failure (109-115), and peripheral arterial disease (117-121).

In this study, improvements in leg vascular insulin sensitivity with the seven-day heating intervention were not accompanied by improvements in femoral artery FMD or a significant de-stiffening of the aorta, likely owing to the

short-term regimen. The observation that improved leg blood flow responses to insulin stimulation following the heating intervention occurred prior to manifestation of other vascular and metabolic changes, stimulates the idea that vascular insulin sensitivity may be a phenotypic attribute that is largely amenable to change. In support of this concept, previous work in genetic models of obesity and diet-induced obesity (139-142) indicate that endothelial insulin resistance is an early event in the disease process that develops prior to manifestation of other indices of vascular endothelial dysfunction (*e.g.*, impaired acetylcholine-induced dilation). Because endothelial insulin resistance is causally implicated in the development and progression of vascular and metabolic disease (14, 17, 18, 21, 29), strategies that correct insulin resistance in the vasculature should be deemed of high relevance.

Another interesting finding of the present study is that although arterial pressure did not change after the heating intervention in T2D subjects, it decreased during heating, a response that was further amplified by the last session. In relation to this finding, recent data show that blood pressure during heating is well maintained in young healthy subjects and that this is due to increased sympathetic nerve activity (247)**.** Conversely, older adults demonstrate a fall in blood pressure with heating, similar to the observation in subjects with T2D, and this fall in blood pressure does not lead to compensatory (*i.e.*, baroreflex-mediated) increases in sympathetic nerve activity (247)**.** Accordingly, it is likely that the drop in blood pressure during heating in older adults and subjects with T2D is mediated by a sympathoinhibitory effect that

alters the compensatory neural response to hypotension (247). The fact that this blood pressure lowering effect of heating in T2D is augmented with more sessions of heating may be attributed to such sympathoinhibitory effects now compounded with improved peripheral vasodilation. Supporting this, eight weeks of passive heating sessions has been shown to decrease sympathetic nerve activity and arterial blood pressure in women with polycystic ovary syndrome (123). Additional studies are warranted to further elucidate the mechanisms and implications of such blood pressure effects of acute and chronic heating.

In summary, the present data generated across several experimental models (*i.e.*, in isolated arteries from animals, *in vivo* in humans, and in cultured endothelial cells) do not support that reduced HSP72 is a key determining factor of endothelial insulin resistance in T2D. Nevertheless, data from this study provides the first evidence that passive heating may be a promising strategy for treating T2D-associated endothelial insulin resistance in the peripheral vasculature.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Figures

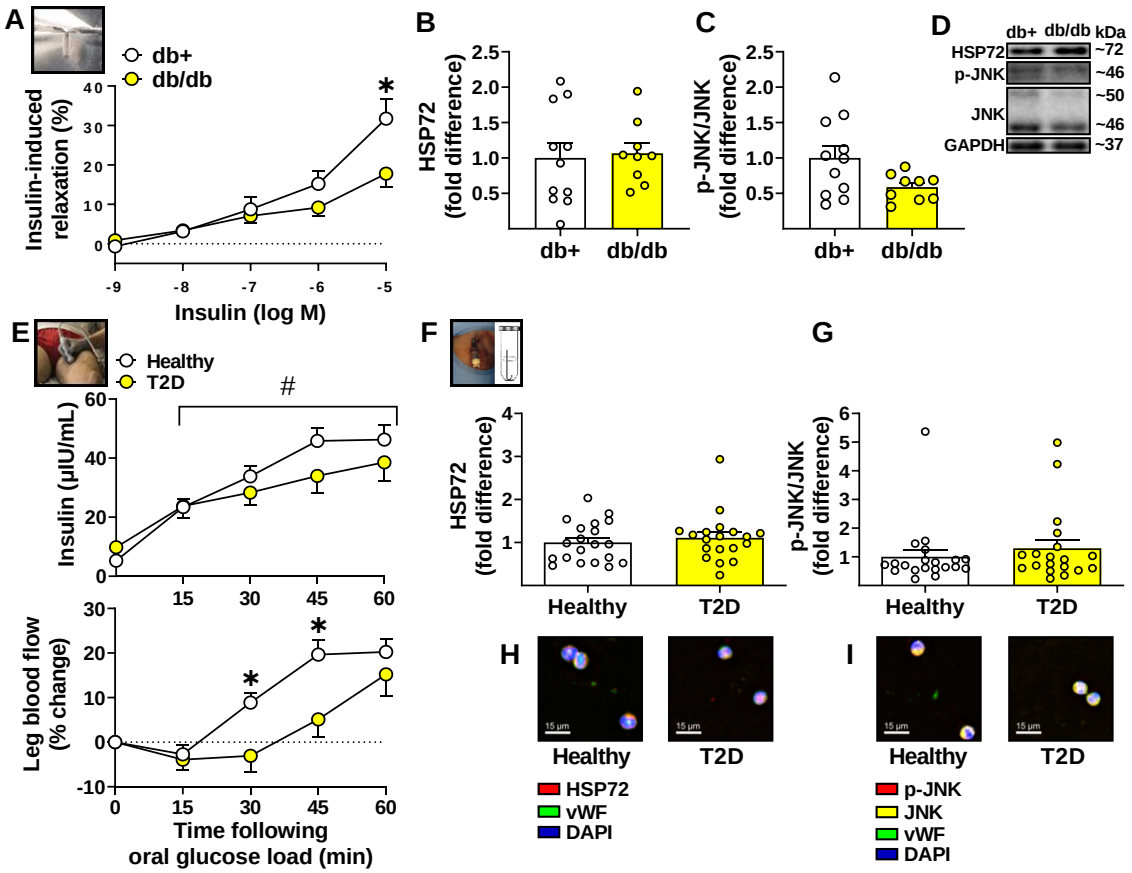

Figure 3.1 Endothelial insulin resistance in T2D mice and humans is not associated with reduced HSP72 in aortas and endothelial cells, respectively. (A) Insulin-induced relaxation, via wire-myography, in aortic rings collected from male db+ and db/db mice (db+ n=11; db/db n=7). Two-way ANOVA with repeated measures and Bonferroni post hoc test. (B) HSP72 and (C) phospho-JNK/JNK protein content in aortas from male db+ and db/db mice, measured by western blotting (db+ n=11; db/db n=9). Unpaired two-tailed student's t test, respectively. (D) Representative western blot bands for proteins of interest in mouse aortas. (E) Plasma insulin concentration (top panel; healthy n=20; T2D n=18), and relative leg blood flow (percent change from baseline) (bottom panel; healthy n=20; T2D n=20) in response to an oral glucose load (75g) in healthy and T2D subjects. Two-way ANOVA with repeated measures and Bonferroni post hoc test. (F) HSP72 and (G) phospho-JNK/JNK protein content in venous endothelial cells collected from healthy and T2D subjects, measured by quantitative immunofluorescence intensity (healthy n=20; T2D n=19). Unpaired two-tailed student's t test, respectively. (H & I) Representative images of quantitative immunofluorescence intensities of HSP72 and phospho-JNK/JNK, respectively. Means ± SEM and individual data, when appropriate, are

reported. In A and E (bottom panel), *denotes significance using Bonferroni-adjusted pairwise comparisons ($P<0.05$). In E (top panel), #denotes significant main effect of time ($P<0.05$).

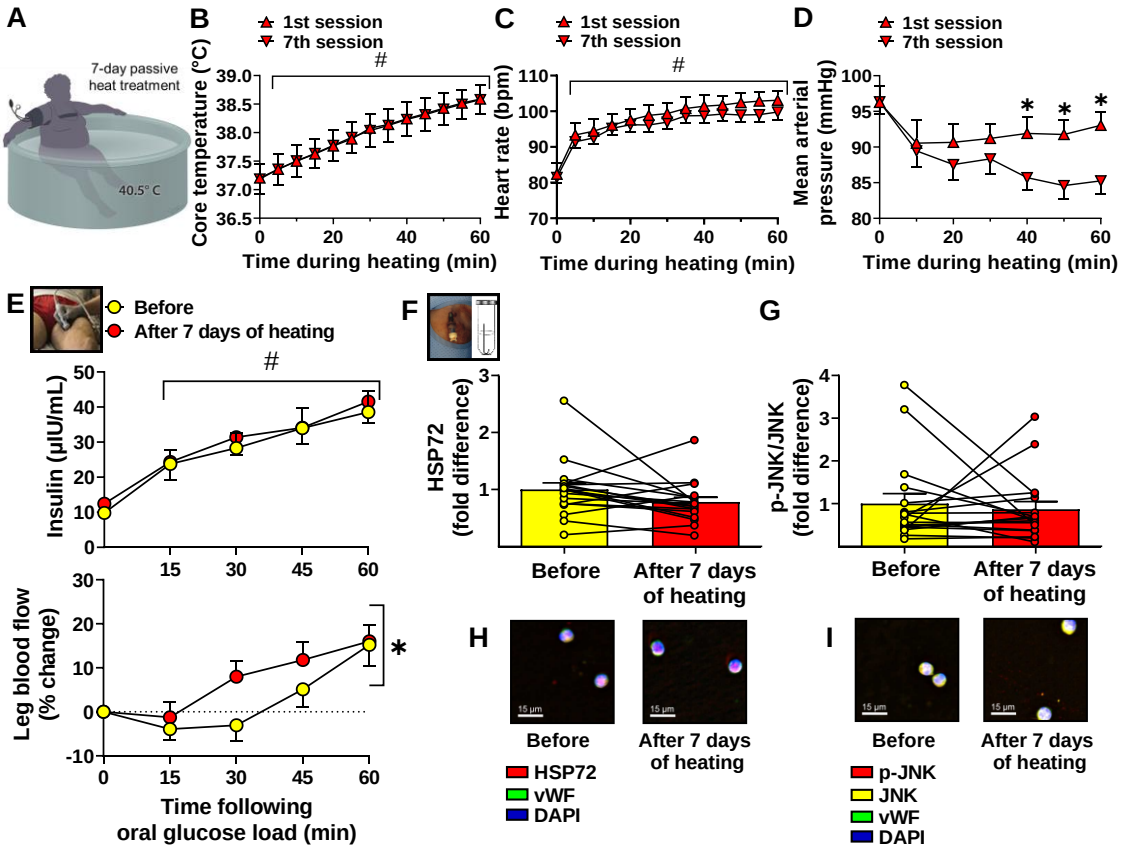

Figure 3.2 Improved leg blood flow responses to an oral glucose load after passive heat treatment does not parallel with increased HSP72 in endothelial cells. (A) Illustration of a single heating session during the seven-day intervention in which a T2D subject is immersed at waist-level in 40.5°C water for 60 minutes. (B) Core temperature, (C) heart rate, and (D) mean arterial pressure measured during the 1st and 7th heating sessions. Two-way ANOVA with repeated measures and Bonferroni post hoc test, respectively. (E) Plasma insulin concentration (top panel; n=18), and relative leg blood flow (percent change from baseline) (bottom panel; n=20) in response to an oral glucose load (75g) in T2D subjects, before and after the seven days of heating. Two-way ANOVA with repeated measures and Bonferroni post hoc test. (F) HSP72 and (G) phospho-JNK/JNK protein content in venous endothelial cells collected from T2D subjects before and after the seven days of heating, measured by quantitative immunofluorescence intensity (n=18). Paired two-tailed student's t test, respectively. (H & I) Representative images of quantitative immunofluorescence intensities of HSP72 and phospho-JNK/JNK, respectively. Means ± SEM and individual data, when appropriate, are reported. In B and C, #denotes significant main effect of time during heating (P<0.05). In D, *denotes significant Bonferroni-adjusted pairwise comparisons (P<0.05). In E (top panel),

#denotes significant main effect of time. In E (bottom panel), *denotes significant main effect of heat treatment (P<0.05).

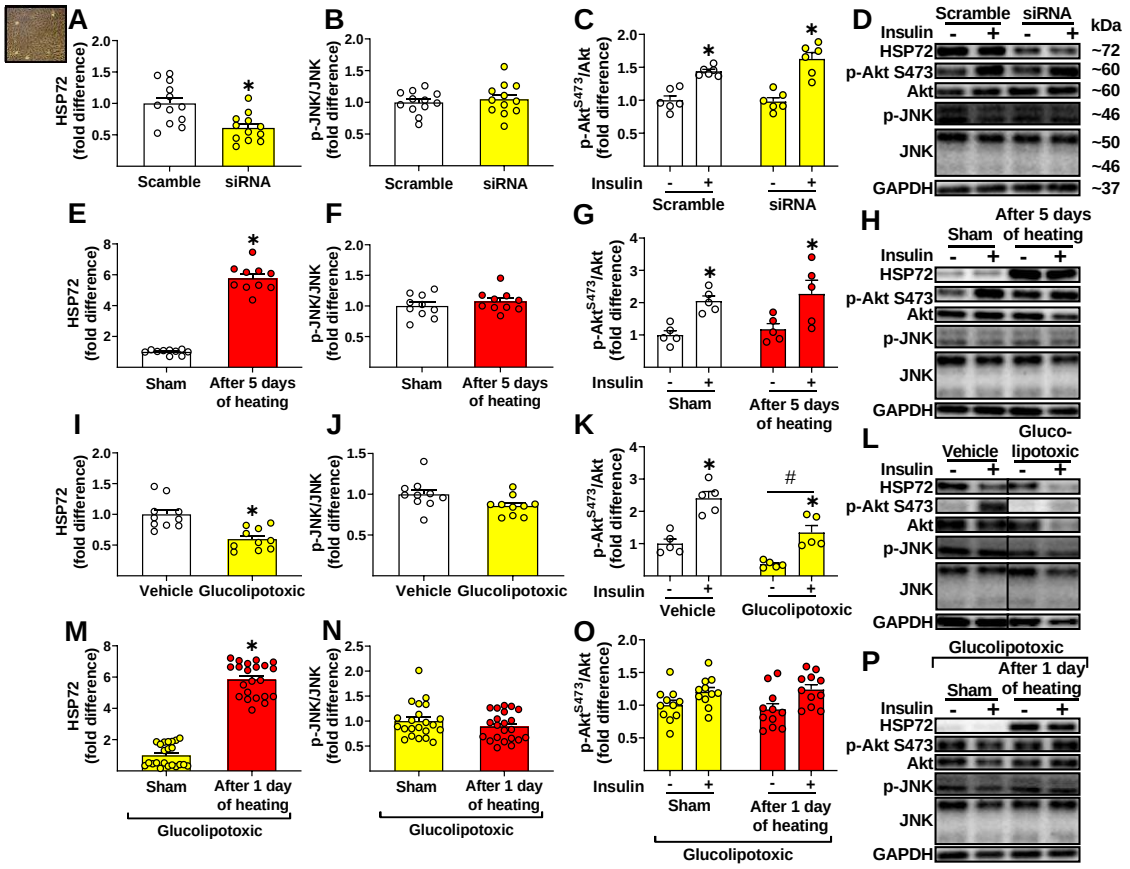

Figure 3.3 Downregulation of HSP72 (via small-interfering RNA) or upregulation HSP72 (via heating) in cultured endothelial cells does not impair and enhance insulin signaling (*i.e.*, activation of Akt), respectively, nor is JNK activity altered. (A) HSP72, (B) phospho-JNK/JNK and (C) insulin-stimulated phospho-AktS473/Akt expression in human skeletal muscle microvascular endothelial cells (hSMMECs), 48 hours after 2nd transfection with scramble or HSP72 siRNA (20nM for 6hr) (A, n=12/condition; B, n= 12/condition; C, n=6/condition). (E) HSP72, (F) phospho-JNK/JNK and (G) insulin-stimulated phospho-AktS473/Akt expression in hSMMECs, 24 hours after sham (37°C) or heat-treatment (40.5°C) for 60 minutes per day, over five days (E, n=10/condition; F, n=10/condition; G, n=5/condition). (I) HSP72, (J) phospho-JNK/JNK and (K) insulin-stimulated phospho-AktS473/Akt expression in hSMMECs, 24 hours after incubation with a glucolipotoxic or vehicle milieu (I, n=10/condition; J, n=10/condition; K, n=5/condition). (M) HSP72, (N) phospho-JNK/JNK and (O) insulin-stimulated phospho-AktS473/Akt expression in glucolipotoxic-treated hSMMECs, 24 hours after sham (37°C) or heat-treatment (40.5°C) for 60 minutes (M, n=22/condition; N, n=22/condition; O, n=11/condition). (D, H, L, and P) Representative western blot bands for proteins of interest (bands in L were obtained from the same gel but are noncontiguous, as denoted by separated boxes). Cells were incubated with insulin (100nM) for

30 minutes prior to collection. Comparisons of HSP72 (A, E, I, M) and phospho-JNK/JNK (B, F, J, N) between conditions within each experiment were performed with unpaired two-tailed student's t test. Comparisons of phospho-AktS473/Akt (C, G, K, O) between conditions in each experiment were performed with two-way ANOVA and Bonferroni post hoc test. Means ± SEM and individual data are reported. In A, E, I, M, *denotes significant effect of condition ($P<0.05$). In C, G, K, *denotes significant main effect of insulin stimulation ($P<0.05$). In K, #denotes significant main effect of glucolipotoxicity ($P<0.05$).

	Healthy subjects	T2D subjects	*P*-value	n (healthy/T2D)
Age (yr)	52 ± 2.1	53 ± 1.9	0.62	20/20
Sex (Female/Male)	(12/8)	(12/8)	--	20/20
Race (n, %)				
Asian	(0, 0%)	(0, 0%)	--	0/0
Black	(0, 0%)	(5, 25%)	--	0/5
Caucasian/Hispanic	(4, 20%)	(0, 0%)	--	4/0
Caucasian/Non-Hispanic	(16, 80%)	(15, 75%)	--	16/15
Other	(0, 0%)	(0, 0%)	--	0/0
Height (cm)	168 ± 2.5	170 ± 1.9	0.58	20/20
Weight (kg)	67.8 ± 2.9	104.0 ± 4.1	**<0.001**	20/20
BMI (kg/m^2)	23.7 ± 0.5	35.9± 1.2	**<0.001**	20/20
Body Fat (%)	30.0 ± 1.7	39.8 ± 1.8	**<0.001**	20/20
Lean Mass (kg)	48.3 ± 2.7	63.0 ± 3.1	**<0.001**	20/20
VAT (kg)	0.33 ± 0.04	0.83 ± 0.07	**<0.001**	20/20
Android/Gynoid Ratio	0.82 ± 0.03	1.07 ± 0.03	**<0.001**	20/20
Systolic BP (mmHg)	116 ± 2	129 ± 3	**<0.001**	20/20
Diastolic BP (mmHg)	72 ± 2	79 ± 2	**0.005**	20/20
MAP (mmHg)	87 ± 1	96 ± 2	**0.003**	20/20
cfPWV (m/s)	6.1 ± 0.2	8.5 ± 0.5	**<0.001**	20/11
Fasted leg blood flow (mL/min)	153 ± 9.3	186 ± 19.6	0.14	20/20
Fasted blood glucose (mg/dL)	79.3 ± 1.2	106.0 ± 6.9	**<0.001**	20/18
Postprandial glucose 2h iAUC (a.u.)	6045 ± 560	8633 ± 1016	**0.03**	20/18
Fasted insulin (μlU/mL)	5.3 ± 0.6	9.8 ± 1.7	**0.01**	20/18
Postprandial insulin 2h iAUC (a.u.)	3802 ± 457	3106 ± 522	0.32	20/18
HOMA-IR	1.0 ± 0.1	2.8 ± 0.6	**0.006**	20/18
HbA1c (%)	5.2 ± 0.07	6.8 ± 0.2	**<0.001**	20/19
MVPA (minutes/week)	285.0 ± 27.7	88.4 ± 19.4	**<0.001**	20/20
Length of T2D diagnosis (yr)	--	7.4 ± 1.2		0/20

Medications

Alpha-glucosidase inhibitor	--	1
Biguanide	--	17
Dipeptidyl peptidase-IV inhibitor	--	1
Glucagon-like peptide-1 agonist	--	3
Sodium-glucose co-transporter 2 inhibitor	--	3
Sulfonylurea	--	6

Thiazolidinedione	--	1
Aldosterone receptor antagonist	--	3
Angiotensin-converting-enzyme inhibitor	--	7
Angiotensin II receptor antagonist	--	3
Beta blocker	--	2
Calcium channel blocker	--	3
Thiazide	--	2
Fibric acid agent	--	1
HMG-CoA reductase inhibitor	--	13
Omega-3-acid ethyl ester	--	1
Thyroid hormone replacement	--	4
Hormone replacement therapy	--	1
Intrauterine device	2	--
Oral contraceptive	1	--

Table 3.1 Subject characteristics, anthropometrics, hemodynamic measurements, and blood profile parameters in healthy subjects and subjects with type 2 diabetes. Data are presented as mean ± SEM. Sample sizes are presented as n. Significant *P* values are in bold. BMI, body mass index; BP, blood pressure; cfPWV, carotid-femoral pulse wave velocity; HbA1C, hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance; iAUC, incremental area-under-the-curve; MAP, mean arterial pressure; MVPA, moderate-vigorous physical activity; T2D, type 2 diabetes; VAT, visceral adipose tissue; a.u., arbitrary units.

	Healthy subjects	T2D subjects	*P*-value	n (healthy/T2D)
Baseline diameter (cm)	0.63 ± 0.02	0.65 ± 0.03	0.60	20/15
Time-to-peak diameter (s)	74.6 ± 7.7	97.2 ± 9.5	0.07	
Hyperemic shear rate (s^{-1}) AUC (a.u.)	10610 ± 1030	9215 ± 856	0.33	
FMD (%)	4.91 ± 0.56	2.04 ± 0.31	**<0.001**	

Table 3.2 Femoral artery hemodynamics in healthy subjects and subjects with type 2 diabetes. Data are presented as mean ± SEM. Sample sizes are presented as n. Significant *P* values are in bold. AUC, area under the curve; FMD, flow-mediated dilation; T2D, type 2 diabetes.

	Before	After 7 days of heating	*P*-value	n
Age (yr)	53 ± 1.9			20
Sex (Female/Male)	(12/8)			20
Height (cm)	170 ± 1.9	170 ± 1.9	0.20	20
Weight (kg)	104 ± 4.1	104 ± 4.0	0.33	20
BMI (kg/m^2)	35.9 ± 1.2	35.8 ± 1.3	0.32	20
Body Fat (%)	39.8 ± 1.7	40.0 ± 1.7	0.21	20
Lean Mass (kg)	63.0 ± 3.1	62.3 ± 2.8	0.17	20
VAT (kg)	0.83 ± 0.07	0.79 ± 0.07	0.30	20
Android/Gynoid Ratio	1.07 ± 0.03	1.07 ± 0.03	0.98	20
Systolic BP (mmHg)	129 ± 3	127 ± 2	0.27	20
Diastolic BP (mmHg)	79 ± 2	78 ± 2	0.58	20
MAP (mmHg)	96 ± 2	94 ± 2	0.24	20
cfPWV (m/s)	8.5 ± 0.5	7.8 ± 0.3	0.07	11
Fasted leg blood flow (mL/min)	186 ± 19.6	184 ± 19.1	0.71	20
Fasted blood glucose (mg/dL)	106 ± 6.9	108 ± 7.2	0.68	18
Postprandial glucose 2h iAUC (a.u.)	8633 ± 1016	9207 ± 859	0.45	18
Fasted insulin (μIU/mL)	9.8 ± 1.7	12.4 ± 2	0.07	18
Postprandial insulin 2h iAUC (a.u.)	3106 ± 522	3029 ± 461	0.74	18
HOMA-IR	2.8 ± 0.6	3.4 ± 0.6	0.14	18
HbA1c (%)	6.8 ± 0.2	6.8 ± 0.2	>0.99	19
MVPA (minutes/week)	88.4 ± 19.4	86.4 ± 15.4	0.87	20

Table 3.3 Effects of seven days of passive heating on subject characteristics, anthropometrics, hemodynamic measurements, and blood profile parameters in subjects with type 2 diabetes. Data are presented as mean ± SEM. Sample sizes are presented as n. Significant *P* values are in bold. BMI, body mass index; BP, blood pressure; cfPWV, carotid-femoral pulse wave velocity; HbA1C, hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance; iAUC, incremental area-under-the-curve; MAP, mean arterial pressure; MVPA, moderate-vigorous physical activity; T2D, type 2 diabetes; VAT, visceral adipose tissue; a.u., arbitrary units.

	Before	After 7 days of heating	*P*-value	n
Baseline diameter (cm)	0.65 ± 0.03	0.65 ± 0.03	0.48	15
Time-to-peak diameter (s)	97.2 ± 9.5	101±12.7	0.84	
Hyperemic shear rate (s^{-1}) AUC (a.u.)	9215 ± 856	9161 ± 973	0.95	
FMD (%)	2.04 ± 0.3	2.67 ± 0.4	0.17	

Table 3.4 Effects of seven days of passive heating on femoral artery hemodynamics in subjects with type 2 diabetes. Data are presented as mean ± SEM. Sample sizes are presented as n. Significant *P* values are in bold. AUC, area under the curve; FMD, flow-mediated dilation; T2D, type 2 diabetes.

CHAPTER FOUR – SUMMARY, LIMITATIONS & FUTURE DIRECTIONS

Summary

This dissertation tested the overarching hypothesis that endothelial insulin resistance in T2D is attributed to decreased endothelial HSP72, whereas increasing endothelial HSP72 with passive heating would improve endothelial insulin responsiveness in T2D. Furthermore, the potential of HSP72 to affect endothelial insulin signaling may involve modulation of JNK activity, a stress kinase known to disrupt the Pi3K-Akt pathway. Accordingly, this dissertation tested the overarching hypothesis through three specific aims.

Firstly, this dissertation sought to determine if endothelial insulin resistance in T2D is attributed to reduced HSP72 expression. It was hypothesized that endothelial insulin resistance in T2D is attributable to decreased HSP72 expression. Subsequently, two approaches were used to test this hypothesis. The first approach hypothesized that isolated aortas from db/db mice would exhibit blunted insulin-induced relaxation and reduced HSP72 relative to isolated aortas from db+ mice. The second approach hypothesized that human subjects with T2D would display impaired postprandial leg blood flow and decreased HSP72 in collected endothelial cells relative to healthy controls. Contrary to the hypothesis, endothelial insulin resistance in T2D mice and humans was not associated with reduced HSP72 in aortas and endothelial cells, respectively.

Secondly, this dissertation aimed to determine if a passive heating intervention designed to upregulate HSP72 would improve postprandial leg blood flow in subjects with T2D. It was hypothesized that seven days of waist level hot water immersion (one hour/day) would improve leg blood flow responses to an oral glucose load (*i.e.*, endogenous insulin stimulation) in subjects with T2D. Furthermore, this improvement would be accompanied by an increase of HSP72 within collected endothelial cells. Counter to this hypothesis, improved leg blood flow responses to an oral glucose load in individuals with T2D after seven days of passive heating was not paralleled with increased endothelial HSP72.

Lastly, this dissertation attempted to determine if modulation of HSP72 influences endothelial insulin signaling in cell culture. It was hypothesized that downregulating HSP72 in endothelial cell culture would impair insulin signaling, whereas upregulating HSP72 would enhance insulin signaling. Contrary to the hypothesis, downregulation (via siRNA knockdown) or upregulation of HSP72 (via heating) in cultured endothelial cells did not impair or enhance insulin signaling, as demonstrated by insulin-stimulated Akt activity. Further supporting the dissociation between HSP72 and endothelial insulin signaling is that modulation of HSP72 did not influence JNK activity, a known stress-activated kinase shown to disrupt the PI3K-Akt insulin-signaling pathway (40-42). The finding that modulation of HSP72 in endothelial cells did not influence JNK activity suggests the link between HSP72 and JNK, supported by other studies in metabolically-active cells and tissues (44-47, 56-58, 61, 63, 75, 76), may not extrapolate to the endothelium.

Collectively, this dissertation demonstrates that HSP72 is not a key determinant of endothelial insulin resistance in T2D. However, passive heating may be an effective treatment for improving endothelial insulin responsiveness in subjects with T2D by a mechanism independent of HSP72. Although speculative, a possible mechanism for improvement in postprandial leg blood flow in subjects with T2D following a passive heating intervention could be mediated by repeated bouts of passive heating increasing endothelial shear stress, rendering the vasculature more responsive to insulin. This dissertation expands upon previous work demonstrating that passive heating augments insulin-stimulated blood flow and perfusion of skeletal muscle in healthy subjects (128). Furthermore, in cultured endothelial cells and isolated arteries applying shear stress for one hour subsequently augmented insulin signaling and insulin-induced dilation, respectively (128). Therefore, passive heating may be a promising therapy for patients with T2D. Future studies are needed to determine if passive heating has other beneficial effects on T2D and the mechanisms responsible for these improvements.

Limitations

The limitations of the dissertation are presented concerning each experimental protocol.

Experimental protocol in isolated aorta from mice

Lack of female mice, resistance arteries, and passive heating

Due to availability, isolated aortas from male db+ and db/db mice were used, presenting several limitations. Firstly, the mouse model used did not match the sex composition of the human cohort, which was mainly female. However, no sex differences were detected on outcome variables within the human protocol.

Secondly, the aorta used in the mouse model did not parallel the use of the superficial femoral artery in human subjects. Expanding this point, insulin may have distinct actions on vessel types due to their location within the arterial tree. Insulin acts on the capillary beds and resistance arteries to increase the delivery of insulin and glucose for glucose uptake (18). Whereas the actions of insulin on conduit arteries such as the aorta may be more to maintain vascular health and protect against atherosclerosis (18). Nevertheless, the peripheral vasculature, such as in the legs, is also prone to atherosclerosis, especially in T2D (248). Ideally, the Inclusion of resistance arteries in conjunction with aortas would have given a global perspective of endothelial insulin resistance for this dissertation.

In line with matching experimental approaches between the mouse model and humans, passive heating could have been applied to mice or isolated arteries. This could have provided more mechanistic insights into the impact of passive heating on endothelial insulin responsiveness that would have been limited to in the human cohort. Collectively, future studies could utilize resistance arteries in conjunction with isolated aortas to assess the potential impact of

passive heating in mitigating insulin-resistance-induced atherosclerosis and impaired insulin-induced relaxation.

Experimental protocol in human subjects

Failure to detect upregulation of HSP72 in endothelial cells from subjects with T2D in response to passive heating

In this dissertation, seven days of passive heating did not increase HSP72 in endothelial cells from subjects with T2D, despite the intervention being designed to upregulate HSP72. The failure to detect any change is unclear but could be due to several factors discussed below.

Subject arms were not always immersed

The most straightforward answer for the failed induction may be that subjects did not immerse their arms. Despite encouragement to immerse their arms whenever possible, subjects were allowed to rest their arms on the sides of the hot tub and out of the water. The allowance for arms to rest on the side of the hot tub served two purposes, first as a safety feature to aid heat dissipation if subjects required it, secondly, to promote intervention adherence by prefacing subject comfort.

The proximity of endothelial cell collection to the heating stimulus

Endothelial cell collection was confined to venous endothelial cells from the arm. Heat-induced HSP72 expression could have been more pronounced in endothelial cells collected from the lower limbs, which were directly heated. However, several research groups have used the collection of venous endothelial

cells from the arm to examine endothelial protein expression to aid subject comfort and logistical purposes (42, 243, 244, 249). Furthermore, venous endothelial cells are an appropriate surrogate for arterial endothelial cells in measuring endothelial protein expression (249) as this approach reduces the risk and invasiveness of collecting arterial samples. Notably, hot water immersion has been shown to increase HSP72 in tissues that are not within the immediate proximity of the heating stimulus, such as the brain in rodents (178), likely due to increased core temperature, which has been cited as a primary stimulus to induce HSPP72 (204). Therefore, it was reasonable to postulate that the heating intervention would increase HSP72 expression in the arms despite not being directly heated.

Upregulating HSP72 with passive heating in humans may be difficult

Passive heating studies designed to increase HSP72 in humans have had varied responses concerning HSP72 induction. Lower body heating with water perfused pants increased HSP72 mRNA in skeletal muscle (105), and localized heating with diathermy increased skeletal muscle HSP72 protein content (103, 104). A single session of water immersion (39°C, 2 hours) increased HSP72 protein in immune cells (102). However, in studies showing regular passive heating improved insulin sensitivity, this improvement occurred without any changes in HSP72 expression in biopsied adipose tissue (125), biopsied skeletal muscle (127), or collected immune cells (126).

Could the lack of HSP72 induction be related to core temperature?

Increased core temperature has often been cited as a primary stimulus for increasing HSP72 expression (204). Gibson *et al*. (80) showed that increasing HSP72 mRNA in immune cells of exercising humans was dependent on increasing core temperature 1-1.5°C, with more robust induction at ~38.5°C the longer this core temperature was maintained. Based on these observations, key studies showing chronic passive heating improves insulin sensitivity and cardiovascular risk profiles were explicitly designed and meticulously executed to increase core temperature to 38.5°C via clavicle level hot water immersion and maintain that core temperature for an hour in an attempt to upregulate HSPs (122, 123, 125). Of note, isolated limb heating with unilateral water perfused pants increased HSP72 mRNA in skeletal muscle without increases in core temperature (105), therefore increased temperature and not specifically increased core temperature may be a requirement to increase HSP72.

Subsequently, this dissertation aimed to upregulate HSP72 in subjects by increasing subject core temperature by 1-1.5°C using waist level hot water immersion, which was selected for two reasons. Firstly, this approach can increase core temperature by 1-1.5°C (198, 216). Secondly, this approach was selected to ensure participant safety, tolerance, and adherence, as subjects with T2D may have impaired thermoregulatory responses to heat, such as impaired sweating and cutaneous vasodilation that increase susceptibility to heat stress (250-253).

Although Ely *et al*. (125) “clamped” subject core temperature at 38.5°C during clavicle level hot water immersion throughout the eight-week intervention,

HSP72 was not increased within adipose tissue. Subsequent studies aiming to increase core temperature by 1-1.5°C to increase HSP72 also saw no change in skeletal muscle (127) or immune cells (126) after 10-days of passive heating. These human data contrast numerous animal studies showing that passive heating increases HSP72 and subsequently improves insulin sensitivity (57, 61, 63, 70, 72-75). This contrast could be due to animals being safely exposed to higher temperatures that could promote heat stress in humans (*i.e.,* reaching a core temperature ≥39.5°C).

Insulin resistance may blunt heat-induced HSP72 expression

Previous studies have shown that exercise-induced (71) and heat-induced (62) HSP72 expression in metabolically-active tissues are restricted in rat models of insulin resistance. In this context, it is plausible that T2D is associated with a deficiency in the heat stress response (48, 49). This may have contributed to the lack of upregulation of HSP72 with heating. However, numerous studies have shown increased HSP72 after passive heating in insulin-resistant rodents (57, 61, 63, 64, 72-75). Therefore, it is unclear if insulin resistance per se contributed to the failed induction of HSP72. Furthermore, future studies are required to determine if heat-mediated induction is blunted in humans with T2D.

No sham-treatment condition with thermoneutral water immersion

The study design did not include a sham-treatment condition (*i.e*., immersion in thermoneutral water). Water immersion can exert hydrostatic

pressure on the body that can have independent effects on cardiovascular function, such as by increasing cardiac output (205, 206), mean arterial blood pressure (206), conduit artery diameter (206), and arterial compliance (205), potentially increasing endothelial shear stress. Therefore, a sham-treatment condition would have allowed for an accurate evaluation of heating effects. However, it has been demonstrated in young and old subjects that lower leg immersion in hot water (42°C) increased shear stress whereas thermoneutral water immersion did not (33°C) (199). Nevertheless, the possible effect of hydrostatic pressure on increasing endothelial shear stress cannot be definitively ruled out.

Lack of direct measurements of endothelial shear stress during passive heating sessions

Based on the finding that HSP72 was not associated with the heat-mediated improvements in postprandial leg blood flow, it is hypothesized that the improvements were likely due to increased endothelial shear stress during heating sessions. However, this study did not collect direct blood flow and shear stress measurements in subjects during hot water immersion. Nevertheless, other measurements during heating may support this hypothesis as heart rate was increased during immersion. Furthermore, it is well documented that hot water immersion increases shear stress (118, 128, 198, 199, 203).

Experimental protocol in human skeletal muscle microvascular endothelial cells (hSMMECs) in culture

Glucolipotoxic-treated cells were limited to a single session of heating

The five days of repeated heating used with healthy cells was selected as an attempt to mimic the seven days of heating experienced by human subjects. This gain of function model did not augment endothelial insulin signaling in healthy cells. However, it may be possible that augmentation of endothelial insulin signaling did not occur as a result of a "ceiling effect" in cells deemed healthy, yet this is only speculative. This five-day heating model was attempted with cells treated with the glucolipotoxic milieu after a previous experiment showed that glucolipotoxic exposure for twenty-four hours could reduce HSP72 expression. These glucolipotoxic treated cells did not survive past the third day of glucolipotoxicity exposure. Therefore, the experiment was amended to assess the impact of a single heating session on glucolipotoxic treated cells based on findings that a single heating session (41°C) increased HSP72 and improved insulin-stimulated Akt activation in isolated skeletal muscle from aged insulin-resistant rats (75).

Future directions

Determine if passive heat-mediated improvements in postprandial leg blood flow were due to increased shear stress experienced during heating sessions

A series of studies have demonstrated that shear stress is essential for arterial adaptation to exercise and passive heating (214-218). In these studies, one arm was occluded with a blood pressure cuff during the intervention, preventing blood flow and shear stress from increasing above resting levels, whereas the other arm was allowed to adapt. Subsequently, arterial adaptations were prevented in the occluded arm following eight weeks of local arm heating (214), waist level hot water immersion (215, 216), lower limb exercise training (217), and handgrip exercise training (218). A similar experimental design could be applied to subjects with T2D to assess if the improvements observed in postprandial leg blood flow following the heating intervention were shear-stress mediated.

Determine the mechanisms for passive heating mediated reductions in arterial blood pressure

Another interesting finding of this dissertation was that although arterial pressure did not change after the heating intervention in T2D subjects, it decreased during heating, a response that was further amplified by the last session. Recent data show that blood pressure during heating is well maintained in young, healthy subjects and that this is due to increased sympathetic nerve activity (247). Conversely, older adults demonstrate a fall in blood pressure with heating, similar to the observation in subjects with T2D, and this fall in blood pressure does not lead to compensatory (*i.e.*, baroreflex-mediated) increases in sympathetic nerve activity (247). Accordingly, it is likely that the drop in blood

pressure during heating in older adults and T2D subjects is mediated by a sympathoinhibitory effect that alters the compensatory neural response to hypotension (247). The fact that this blood pressure-lowering effect of heating in T2D is augmented with more heating sessions may be attributed to such sympathoinhibitory effects now compounded with improved peripheral vasodilation. Supporting this, eight weeks of passive heating sessions have decreased sympathetic nerve activity and arterial blood pressure in women with PCOS (123). Additional studies are warranted to elucidate further the mechanisms and implications of such blood pressure effects of acute and chronic heating.

Determine if passive heating affects endothelial protein expressions by localizing heating to the arms

As stated in the limitations section, endothelial cell collection was confined to the arms, yet the heating stimulus was applied to the lower body. Endothelial cells collected closer to the heating stimulus could be a better source of information in examining the effects of passive heating on the vasculature. In line with this, isolated arm heating with endothelial cells collection could provide crucial molecular information on the effect of passive heating improving vascular insulin responsiveness.

Evaluate the effect of chronic passive heating on vascular insulin responsiveness in subjects with T2D

Based on the findings that short-term (*i.e.*, seven days) passive heating improved postprandial blood flow in subjects with T2D, suggesting that passive heating improved endothelial insulin responsiveness, it is reasonable to postulate that chronic passive heating could augment improvements. In line with this hypothesis, exercise training has improved insulin-stimulated blood flow and perfusion in patients with T2D (77-79). Of further promise is that chronic passive heating studies have improved insulin sensitivity, sympathetic activity, and indices of vascular function in obese and insulin-resistant subjects that characterize T2D (122, 123, 125). Notably, this dissertation observed a trend towards decreased aortic stiffness as shown by decreased cfPWV after the heating intervention in subjects with T2D ($P = 0.07$, Table 3.2). Chronic passive heating has been shown to reduce aortic stiffness, including in individuals with obesity (122). The clinical relevance is that increased aortic stiffness is associated with an increased risk for CVD in T2D (254, 255). Collectively these data indicate that chronic passive heating may reduce CVD risk in patients with T2D.

Nevertheless, chronic passive heating studies with subjects with T2D are still lacking. Such studies could provide information on the effectiveness of passive heating as a treatment option, which is warranted as passive heating could be a beneficial alternative to exercise for individuals with T2D, who often have reduced cardiorespiratory fitness and a decreased capacity for sustained exercise (92-99). Furthermore, patients with T2D have often cited barriers to exercise related to physical and mental discomfort that perpetuate unmotivated

behaviors (100, 101). Therefore, passive heating could be an appealing treatment for individuals who cannot exercise, are discouraged by it, or choose not to participate.

Determine if a combination therapy of exercise and passive heating augment improvements in vascular insulin responsiveness

Exercise has been shown to improve insulin-stimulated blood flow and perfusion in patients with T2D (77, 78). This dissertation suggests passive heating could be another potential strategy. Exercise and passive heating can induce similar physiological responses and adaptations that promote health benefits (223), with both strategies as effective as each other in inducing beneficial adaptations (117, 224, 225). However, divergent responses exist between the two strategies, as shown by Hesketh *et al.* (222), who showed six weeks of sauna bathing and exercise increased skeletal muscle capillarization and eNOS content to the same extent, yet the exercise specific skeletal muscle adaptations also. It is reasonable to postulate that a combination strategy could be synergistic, potentially augmenting improvements. Recently, it was shown that eight weeks of moderate-intensity walking did not improve endothelial insulin responsiveness in subjects with T2D (33). As such, passive heating could potentially improve endothelial insulin responsiveness by a different mechanism for individuals who might have a low sensitivity to exercise-induced adaptations (256). Future studies are needed to test this potential attractive prospect of combining both therapies for augmented benefits.

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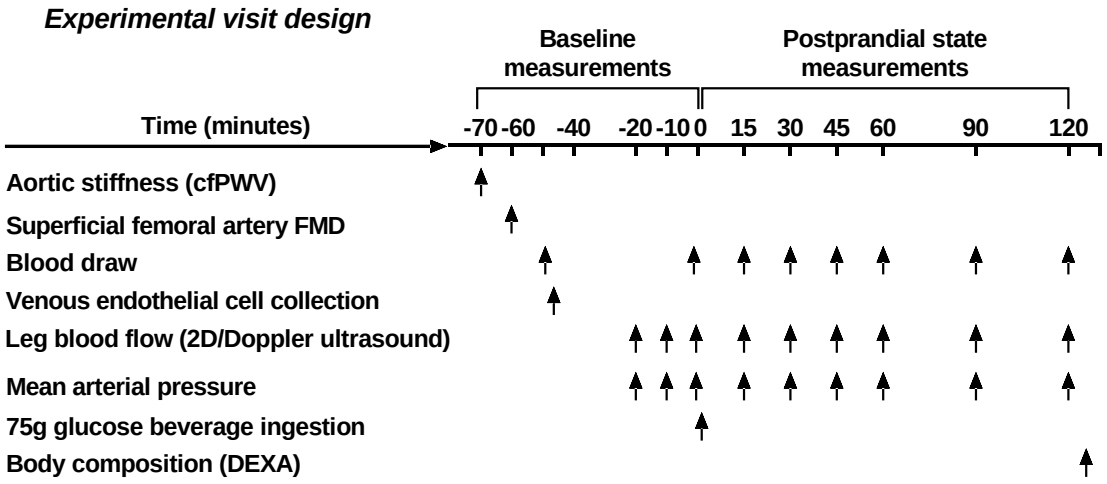
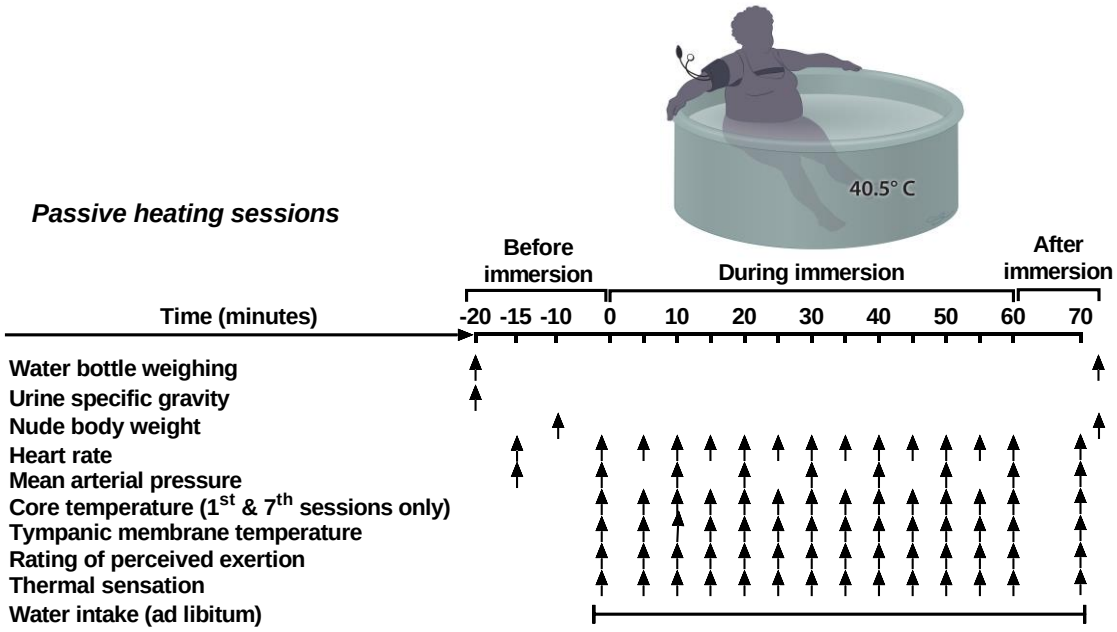
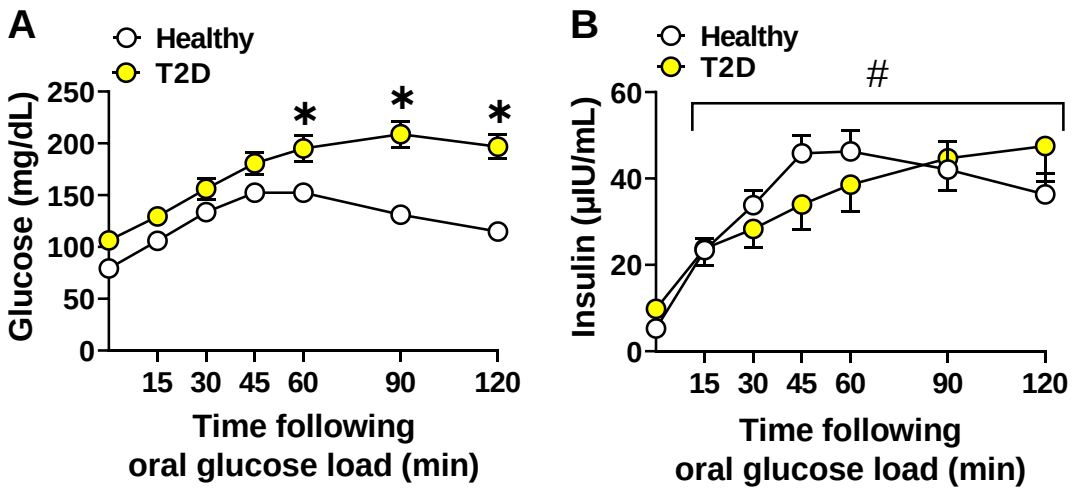
APPENDIX I – CHAPTER THREE SUPPLEMENTAL FIGURES



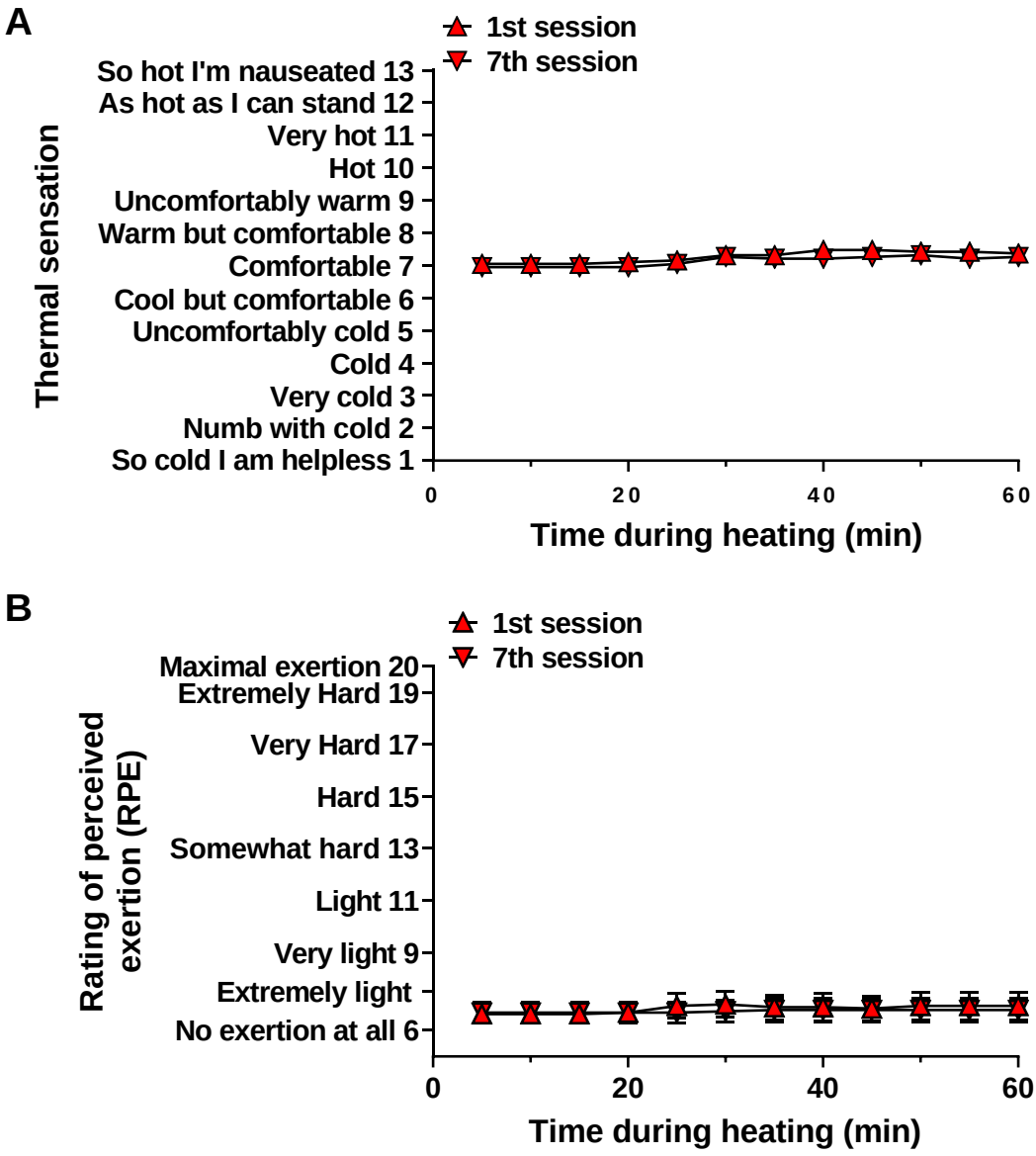
Appendix I Figure 1 Experimental visit design and measurements. cfPWV, carotid-femoral pulse wave velocity; FMD, flow-mediated-dilation; DEXA, dual-energy X-ray absorptiometry.



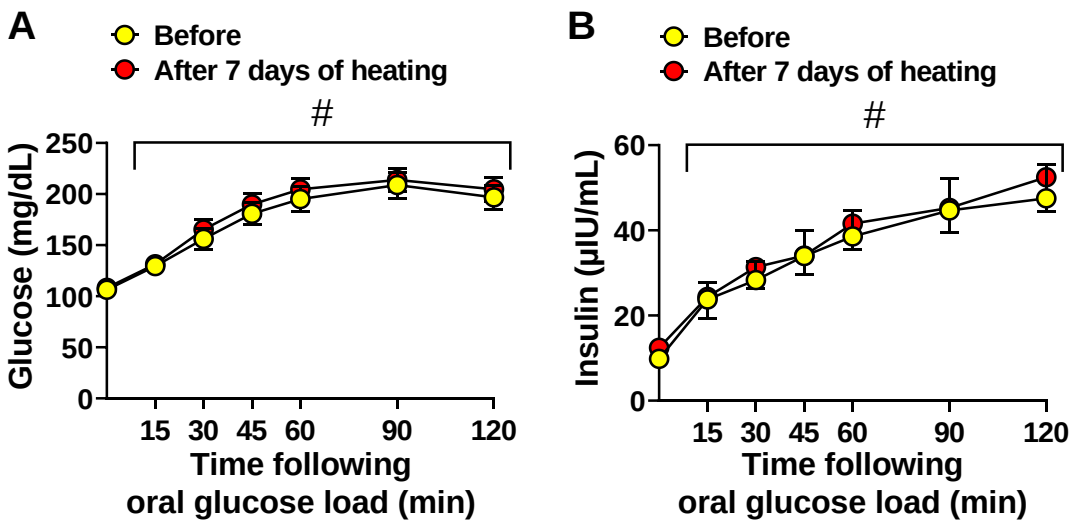
Appendix I Figure 2 Passive heating sessions and measurements.



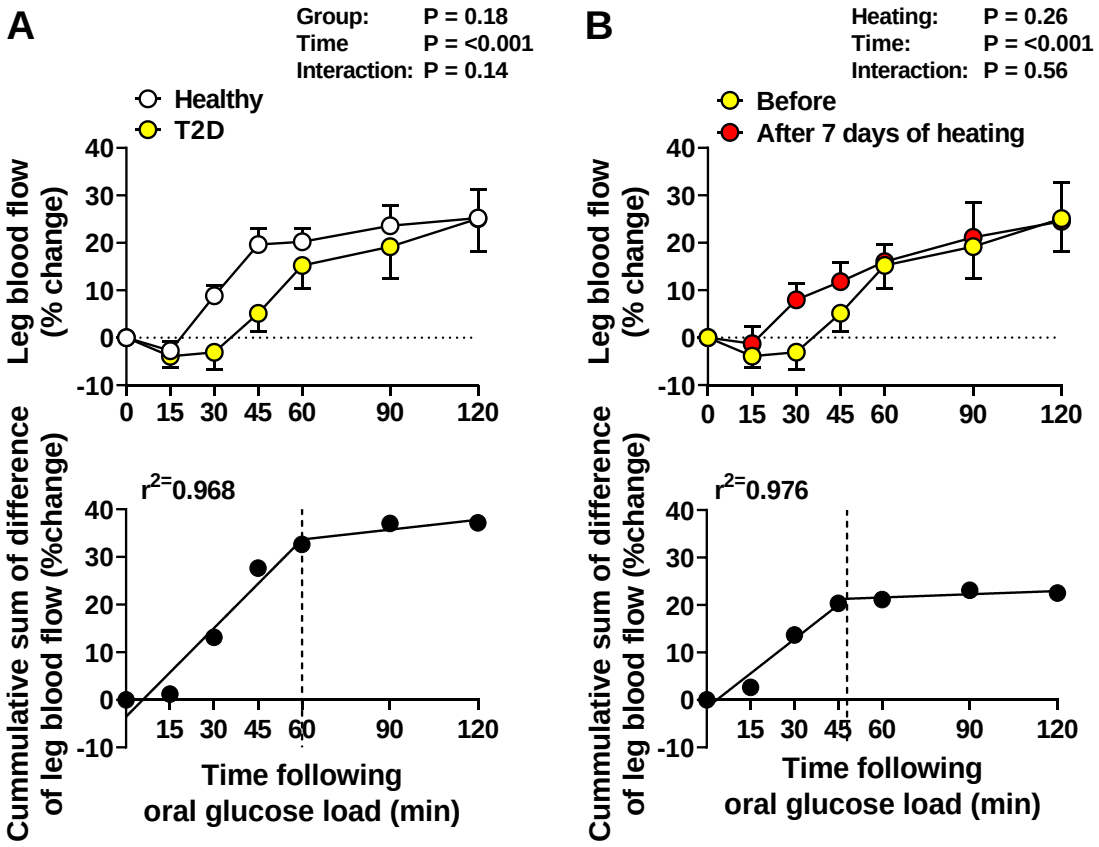
Appendix I Figure 3 Blood glucose and plasma insulin concentrations in response to an oral glucose load between healthy subjects and subjects with T2D. (A) Blood glucose and (B) plasma insulin concentrations in response in response to an oral glucose load (75g) in healthy and T2D subjects (healthy n=20; T2D n=18). Means ± SEM are reported. In A, *denotes significance using Bonferroni-adjusted pairwise comparisons (P<0.05). In B, #denotes significant main effect of time (P<0.05).



Appendix I Figure 4 Thermal sensation and rating of perceived exertion during heating. (A) Thermal sensation responses to the 13-point McGinnis categorical scale and (B) Rating of perceived exertion responses to Borg's 6-20 point scale, measured during the 1st and 7th heating sessions. Two-way ANOVA with repeated measures and Bonferroni post hoc test, respectively. Means ± SEM are reported.



Appendix I Figure 5 Blood glucose and plasma insulin concentrations in response to an oral glucose load in subjects with T2D, before and after the seven days of heating. (A) Blood glucose and (B) plasma insulin concentrations in response to an oral glucose load (75g) in T2D subjects, before and after the seven days of heating (n=18). Two-way ANOVA with repeated measures and Bonferroni post hoc test. Means ± SEM are reported. In A and B, #denotes significant main effect of time ($P<0.05$).



Appendix I Figure 6 Segmental regression curves of the cumulative sum of the difference of relative postprandial leg blood flow indicate detectable differences were limited to within the first hour after ingestion. (A) Relative leg blood flow (percent change from baseline) (top panel; T2D n=18) and corresponding segmental regression curve of the cumulative sum of the difference between healthy subjects and subjects with T2D (bottom panel). (A, top panel) Two-way ANOVA with repeated measures and Bonferroni post hoc test. (B) Relative leg blood flow (percent change from baseline) (top panel; T2D n=18) and corresponding segmental regression curve of the cumulative sum of the difference between before and after seven days of heating (bottom panel). (B, top panel) Two-way ANOVA with repeated measures and Bonferroni post hoc test. Means ± SEM, when appropriate, are reported. In A & B (bottom panels), r^2 represents the goodness of fit value for curve. In A & B (bottom panels) dotted lines indicate the timepoint where slope of the segmental regression curve deviate.

APPENDIX II – INFORMED CONSENT FORM

CONSENT FORM TO PARTICIPATE IN A RESEARCH STUDY

INVESTIGATOR'S NAME: JAUME PADILLA PHD

PROJECT IRB #: 2008181

STUDY TITLE: RESTORING VASODILATOR ACTIONS OF INSULIN IN PATIENTS WITH TYPE 2 DIABETES: (ROLE OF PHYSICAL ACTIVITY IN RESTORING VASCULAR INSULIN SENSITIVITY IN SKELETAL MUSCLE OF PATIENTS WITH TYPE 2 DIABETES).

INTRODUCTION

This consent may contain words that you do not understand. Please ask the investigator or the study staff to explain any words or information that you do not clearly understand.

This is a research study. Research studies include only people who choose to take part. Before participating in a research study, you have the right to know about the study expectations and tests that will be used in this study. This will allow you to make a choice on whether or not to join. The information presented here is simply an effort to make you better informed so that you can make your choice of whether or not to join this research study.

This is a clinical trial and a description of this clinical trial is available on www.ClinicalTrials.gov (#NCT03203694), as required by U.S. law. This web site does not include information that can identify you. At most, the web site includes a summary of the results. You can search this web site at any time.

This study is being sponsored by the University of Missouri, Department of Nutrition and Exercise Physiology.

WHAT SHOULD I KNOW BEFORE I DECIDE WHETHER TO TAKE PART IN THIS STUDY?

- Research studies help us to learn new things and test new ideas about treating certain conditions/diseases.
- Taking part in a research study is voluntary. You decide if you want to take part, and you can stop taking part at any time. Your regular medical care at the University of Missouri Hospitals and Clinics will not be affected now or in the future if you decide you do not want to be in this study.
- We are doing this study to determine the effects of lower body heating on leg vascular function in patients with type 2 diabetes (T2D).
- We invite you to take part in this study because you are between the ages of 35 and 65 years and have type 2 diabetes. Or are between the ages of 35 and 65 and do not have type 2 diabetes
- About 40 people will take part in this study at the University of Missouri.

If you have Type 2 diabetes:

- The study involves two ~4 hour experimental visits that all take place at the University of Missouri Physical Activity and Wellness Center (MUPAW) in the Department of Nutrition and

Exercise Physiology. During the experimental visits we will be performing various measures of arterial and metabolic health. We will explain these procedures in this form. These visits will take place approximately 8 days apart during which you will undergo lower body heating for 60 min, for 7 days at the MU Physical Activity and Wellness (MUPAW) center in the Department of Nutrition and Exercise Physiology. Between your visits, we will also provide you with supplies to use at home that assess your physical activity and dietary intake.

- The total amount of time you could be in this study is about 2 weeks.

If you do not have Type 2 diabetes:

- The study involves a single ~4 hour experimental visit. This will take place at the University of Missouri Physical Activity and Wellness Center (MUPAW) in the Department of Nutrition and Exercise Physiology. Before your visit, we will also provide you with supplies to use at home that assesses your physical activity and dietary intake.
- The total amount of time you could be in this study is about ~1 week.

Overall:

- Taking part in this study may or may not make your health better. We hope that the information we learn from this study will help in the future treatment of people with Type 2 Diabetes. **There is no guarantee that taking part in this research will result in any improvement in your condition.**

- As with any research study, there are risks that we **know about and there may be some we don't** know about. We will explain these risks in this form.

- We will only include you in this study if you give us your permission first by signing this consent form. Please take your time to make your decision and discuss it with your family and friends.

WHAT IS INVOLVED IN THE STUDY?
You will initially be screened to see if you meet the criteria of the study.

Prior to participating in the study, you will provide written informed consent and complete a medical health history questionnaire. You will be scheduled to come to MUPAW for the experimental visits. On testing days, you will arrive in the morning after an overnight fast, having not taken medications, and not exercised for 24-48 hrs. Between your visits we will give you an accelerometer that fits on your waistband to wear for 7-day periods so we can quantify your physical activity. We will also give you 3-day food diaries to fill out.

If you have type 2 diabetes, you will undergo lower body heating for 60 min, for 7 days at the MU Physical Activity and Wellness (MUPAW) center in the Department of Nutrition and Exercise Physiology. Lower body heating will be accomplished via water bath immersion at a temperature of 40.5°C (105 F), using an inflatable hot tub. During the lower body heating, you will be seated, and water will reach a level between your naval (belly button) and armpit. The purpose of the lower body heating is to increase leg blood flow. This water temperature is below the threshold for pain sensation or burning. Your experimental visits will be scheduled for before and after the 7 days of lower body heating sessions.

Between your visits, we will provide you with supplies to use at home that assesses your physical activity and dietary intake.

If you do not have Type 2 diabetes you will participate in a single experimental visit. On the testing day, you will arrive in the morning after an overnight fast, having not taken medications, and not exercised for 24-48 hrs. Before your visit, we will provide you with supplies to use at home that assesses your physical activity and dietary intake.

Experimental Visit: This visit will last approximately 4 hours and will include the following measures/tests:

Basic measures: Body weight and height.

Urine sample: If you are a woman of childbearing potential, you will give a urine sample for a pregnancy test.

Body composition: A DEXA scan will be performed to determine how much of your body is composed of fat, bone and muscle. This scan will expose you to a small amount of radiation. The amount of radiation received during the DEXA scan is less than that of an airline flight from California to New York and back.

Vascular function measures: A blood pressure cuff will be placed around the forearm or below the knee. This cuff will be inflated (250 mmHg), as is done when your blood pressure is being measured, but instead of deflating the cuff immediately it will remain inflated for 5 minutes. We will measure the blood flow to your arm and leg by placing an ultrasound probe over the brachial artery (upper arm artery) and superficial femoral artery (upper leg artery) before, during and after inflating the cuff. The probe will provide a measure of the speed at which your blood is traveling through your artery and the extent to which your artery dilates (i.e., expands). A pressure sensor (tonomter, the size of a pencil) will be placed over the skin of the neck region to obtain the pressure wave form in the carotid artery.

Oral glucose tolerance test: The oral glucose tolerance test helps to determine how well your body disposes of blood sugar (glucose) in response to insulin. You will have a small catheter (tube) placed into a vein in a arm. During the entire test you will lay comfortably in a bed. The total volume of blood drawn per test will be 45mL (~10 teaspoons). During this time, we will perform repeated measures of blood flow in a leg using an ultrasound probe, measures of blood pressure, and measures of heart rate via ECG.

Endothelial cell collection: Cells will be collected from the inside of your vein in which the catheter for the oral glucose tolerance test is placed. Briefly, after the catheter is placed a small wire is inserted to rub the side of the vessel for us to collect these cells. Any discomfort you will feel will be from the initial stick normally felt when the needle goes into the arm to place the catheter; the inside of the vessels will not feel the collection of the cells when we insert the small wires.

Lower body heating sessions: During these sessions you will sit in an inflatable hot tub for 60-minutes with the water level between your belly button and armpit, and the water temperature set at 40.5°C (105°F). All sessions will be supervised. Before and after entering the hot tub, we will measure how hydrated you are by collecting a urine sample from you and from your body weight that you will record

will while not wearing any clothes. A digital scale will automatically record your weight while you are alone in a room getting changed. If the urine sample and body weights indicate that you are dehydrated, we will give you bottled water to drink. While in the hot tub you will be encouraged to drink provided bottled water. While in the hot tub we will measure your body temperature by using a digital ear thermometer that has single-use probes on its tip, that will be placed in your ear for 5-seconds. We will also record your heart rate and blood pressure by fitting you with wearable devices. We will also ask you how warm you feel using a visual scale. During the first and last heating sessions we will measure your core temperature by having you ingest an FDA-approved telemetry pill, 7-9 hours before your session. This pill is a single use device that will pass easily through your digestion system and it is often used in research involving hospital patients, firefighters, and military personnel. If you feel too warm while in the hot tub, you can exit the tub at any time.

HOW LONG WILL I BE IN THE STUDY?
If you have Type 2 diabetes you will be in the study for ~2weeks. If you do not have Type 2 diabetes you will be in the study for ~1 week. You can stop participating at any time. Your decision to withdraw from the study will not affect in any way your medical care and/or benefits.

WHAT ARE THE RISKS OF THE STUDY?
While on the study, you are at risk for the side effects described below. You should discuss these with the investigator and/or your doctor. There may also be other side effects that we cannot predict. Risks and side effects related to the procedures in this study include:

Lower body heating: While we do not expect any adverse events, the potential risks associated with the heating protocol is dehydration and/or the onset of heat stress. To prevent these risks, all sessions are supervised, and you are continuously monitored for signs and symptoms that include lightheadedness, dizziness, weak pulse, nausea, headache, feeling unbearably warm, or a body temperature above 39.5°C. To assess all of these you will have the following performed during each visit: urine sampling, nude body weight recordings, frequent recordings of body temperature, heart rate, blood pressure and perception of how warm you are. If you have signs or symptoms previously described, you are removed from the water bath, placed in a recovery chair with feet elevated, and cooled down with cold packs until body temperature returns to pre-immersion values. If signs or symptoms worsen such as fainting, agitation, confusion, seizures, inability to drink, or body temperature not falling below 39.5°C despite application of cooling packs, immediate medical attention will be sought. To keep your body temperature at 38.5°C, you are seated with the water level reaching between your belly button and armpit. To further prevent dehydration, you will be encouraged to drink provided bottled water while in the water bath. Additionally, if you feel too warm you will be allowed to exit the water bath.
The 40.5°C(105°F) water temperature is well below the threshold for pain sensation or burning. Temperature of the water will be continuously monitored.

Insertion of venous catheters: The potential risks of venous catheterization include infection, swelling and discomfort at the catheter insertion sites. Some bleeding may occur during the insertion of the catheters as well after the catheters have been removed. There is also the possibility of fainting, dizziness, and possible pain and bruising as a result of catheter insertion. These risks will be greatly minimized by using sterile procedures and having an experienced registered nurse placing the venous catheters.

Oral glucose tolerance test: The risks of the OGTT are minimal. This is a regular glucose dose. Some people may feel nausea after drinking the glucose, rarely do people experience light headedness. Trained personnel conduct this study.

Heart rate measurements via ECG: Some people may have a skin irritation from the patches that connect the wires on your chest to the computer. Skin and hair are pulled slightly when the patches are removed after the test. Research personnel will attach and remove the patches as carefully as possible.

Blood pressure cuff inflation and vascular function measures: The blood pressure cuff will squeeze your arm or leg tightly; however, any discomfort will be alleviated as soon as the pressure in the cuff is released.

Body composition: The amount of radiation received during the DEXA scan is less than that of an airline flight from California to New York and back. If you are a women of child-bearing age, you will be administered a home pregnancy test to ensure that you are not pregnant prior to the scan. You will be discontinued from the study if pregnant.

Ingestible telemetry pill for core temperature: Manufacturer instructions detail that the CorTemp® pill **should not be used by individual's that have any of the following: 1) someone less than 80 pounds, 2)** someone diagnosed with but not limited to diverticulitis, inflammatory bowel disease, gag reflex disorders or impairments, previous gastrointestinal surgery, hypomotility of the gastrointestinal tract (such as Ileus), 3) someone undergoing Nuclear Magnetic Resonance (NMR) or MRI scanning while the CorTemp® is within the body,4) someone having a cardiac pacemaker or other implanted electro medical device, 5) people who have difficulty swallowing. Therefore, if you have anything from the previous list, you will be excluded from the study.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?
If you agree to take part in this study, there may or may not be direct medical benefit to you. You may expect to benefit from taking part in this research to the extent that you are contributing to medical knowledge. Indeed, we hope the information learned from this study will lead to future strategies to combat cardiovascular disease. You may benefit from the proposed study by learning more about your cardiovascular and metabolic health. In addition, you may also benefit from the lower body heating intervention designed to improve vascular function.

WHAT OTHER OPTIONS ARE THERE?
An alternative is to not participate in this research study. At any point, you can change your mind about being in the study.

WHAT ABOUT CONFIDENTIALITY?
Information produced **by this study will be stored in the investigator's file and identified by a code** number only. The code key connecting your name to specific information about you will be kept in a separate, secure location. Information contained in your records may not be given to anyone unaffiliated with the study in a form that could identify you without your written consent, except as required by law. It is possible that your medical and/or research record, including sensitive information and/or identifying information, may be inspected and/or copied by the study sponsor (and/or its agent), the Food and Drug Administration (FDA), federal or state government agencies, or hospital accrediting agencies, in the course of carrying out their duties. If your record is inspected or copied by the study sponsor (and/or its

agents), or by any of these agencies, the University of Missouri will use reasonable efforts to protect your privacy and the confidentiality of your medical information.

The results of this study may be published in a medical book or journal or used for teaching purposes. However, your name or other identifying information will not be used in any publication or teaching materials without your specific permission.

We will keep the information we collect from you for this study to use in future research/to share with other investigators to use in future studies without asking for your consent again. Information that could identify you will be removed from your research data so no one will know that it belongs to you.

WHAT ARE THE COSTS?
You will not be charged for any of the tests or procedures done for the research study. You will be responsible for your travel to and from the laboratory/testing facilities.

WILL I BE PAID FOR PARTICIPATING IN THE STUDY?
If you are receiving the lower body heating sessions, you will be compensated $800 for completion of the duration of the study.

If you are part of the reference group participating in the single aerobic test and experimental visit you will be compensated $200.

If you or the investigator discontinue the study, you will be compensated $15 for every hour spent in the laboratory/testing facility.

The method of payment will be check.

We will need your social security number in order to pay you. Any payment may need to be reported as income on your tax return. If you are not a resident/citizen (non-resident alien) of the United States, you will need to work with the MU Nonresident Tax Specialist at 573-882-5509.

WHAT IF I AM INJURED?
It is not the policy of the University of Missouri to compensate human subjects in the event the research results in injury. The University of Missouri, in fulfilling its public responsibility, has provided medical, professional and general liability insurance coverage for any injury in the event such injury is caused by the negligence of the University of Missouri, its faculty and staff. The University of Missouri also will provide, within the limitations of the laws of the State of Missouri, facilities and medical attention to subjects who suffer injuries while participating in the research projects of the University of Missouri. In the event you have suffered injury as the result of participation in this research program, you are to contact the Risk Management Officer, telephone number (573) 882-1181, at the Health Sciences Center, who can review the matter and provide further information. This statement is not to be construed as an admission of liability.

WHAT ARE MY RIGHTS AS A PARTICIPANT?
Participation in this study is voluntary. You do not have to participate in this study. Your present or future care will not be affected should you choose not to participate. If you decide to participate, you can change your mind and drop out of the study at any time without affecting your present or future care in the University of Missouri Health Care System. Leaving the study will not result in any penalty

or loss of benefits to which you are entitled. In addition, the investigator of this study may decide to end your participation in this study at any time after he/she has explained the reasons for doing so and has helped arrange for your continued care by your own doctor, if needed.

You will be informed of any significant new findings discovered during the course of this study that might influence your health, welfare, or willingness to continue participation in this study.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?
If you want to talk privately about your rights or any issues related to your participation in this study, you can contact University of Missouri Research Participant Advocacy by calling 888-280-5002 (a free call), or emailing MUResearchRPA@missouri.edu.

PARTICIPANT DATABASE OPTION

WHAT INFORMATION IS COLLECTED IN THE DATABASE?

You are being asked to grant permission to review your screening file and enter some of your personal and medical information in a computer database (spreadsheet). The following information will be collected: Demographics, study-relevant diagnoses (i.e., diabetes, heart disease, etc.); relevant treatment history (medications, surgeries, lifestyle or other interventions, etc.); and basic contact information together or separate from your study information as requested. The information will be placed in a **"database," which will be kept on file indefinitely. This file will be maintained within th**e secured server of the Department of Nutrition and Exercise Physiology. Access will only be granted to investigators within the department who are approved to conduct associated human health research studies.

It is anticipated that information from the database will be used to conduct research projects and eventually help improve the quality of information upon which future treatment decisions are based. These include reviewing the information for patterns or extracting pieces of information to look at more closely in order to understand the course of disease or the effect of particular treatments. Investigators within the Department of Nutrition and Exercise Physiology may contact you in the future (if you select this option below) to participate in additional research studies for which you may qualify. You are free to decline participation in such projects even after your information is entered into the database. You may also opt out of future contact at any time by notifying us in writing.

PERMISSIONS FOR USE OF YOUR INFORMATION

Please indicate your choice about research being performed on your database information by answering the questions below.

Do you give your permission for your information to be entered in the database and to be used for anonymous research, where no identifying information will be released?

Yes _____ No _____ Initials _____

Do you give your permission to be contacted in the future and asked for your consent to participate in any research project which would require identifying information to be released to the investigators?

Yes _____ No _____ Initials _____

Do you give your permission to be contacted about possible participation in future research projects that may grow out of research on the database information?

Yes _____ No _____ Initials _____

You may ask more questions about the study at any time. For questions about the study or a research-related injury, contact Dr. Jaume Padilla at padillaja@missouri.edu or (812) 345-3566.

A copy of this consent form will be given to you to keep.

I authorize the investigators to keep this information and any information from my participation in their studies in a database so that they may contact me regarding future studies.

YES _____ **NO** _____

Are you participating in any other research projects? Yes _____ No _____

SIGNATURE

I confirm that the purpose of the research, the study procedures, the possible risks and discomforts as well as potential benefits that I may experience have been explained to me. Alternatives to my participation in the study also have been discussed. I have read this consent form and my questions have been answered. My signature below indicates my willingness to participate in this study.

_____ _____
Subject Date

_____ _____
Witness (if required) Date

SIGNATURE OF STUDY REPRESENTATIVE

I have explained the purpose of the research, the study procedures, identifying those that are investigational, the possible risks and discomforts as well as potential benefits and have answered questions regarding the study to the best of my ability.

_____ _____
Study Representative* Date

*Study Representative is a person authorized to obtain consent. Per the policies of the University of Missouri Health Care, for any 'significant risk/treatment' study, the Study Representative must be a physician who is either the Principal or Co-Investigator. If the study is deemed either 'significant risk/non-treatment' or 'minimal risk,' the Study Representative may be a non-physician study investigator.

APPENDIX III – ABSTRACTS OF PEER-REVIEWED PUBLICATIONS

First author publications (1)

Randomized Controlled Trial > Obesity (Silver Spring). 2021 Jul;29(7):1146-1154.
doi: 10.1002/oby.23173.

Leg Fidgeting During Prolonged Sitting Improves Postprandial Glycemic Control in People with Obesity

Ryan J Pettit-Mee [1,2], Sean T Ready [1], Jaume Padilla [1,2], Jill A Kanaley [1]

Affiliations + expand

PMID: 34159757 PMCID: PMC8231734 (available on 2022-07-01) DOI: 10.1002/oby.23173

Abstract

Objective: Studies have shown that fidgeting augments metabolic demand and increases blood flow to the moving limbs, whereas prolonged sitting suppresses these factors and exacerbates postprandial glucose excursions. Therefore, the hypothesis of this study was that leg fidgeting during prolonged sitting would improve postprandial glycemic control.

Methods: Adults with obesity (n = 20) participated in a randomized crossover trial in which blood glucose and insulin concentrations were measured during a 3-hour sitting period following the ingestion of a glucose load (75 g). During sitting, participants either remained stationary or intermittently fidgeted both legs (2.5 minutes off and 2.5 minutes on). Accelerometer counts, oxygen consumption, and popliteal-artery blood flow were also measured during the sitting period.

Results: As expected, fidgeting increased accelerometer counts ($P < 0.01$), oxygen consumption ($P < 0.01$), and blood flow through the popliteal artery ($P < 0.05$). Notably, fidgeting lowered both glucose ($P < 0.01$) and insulin ($P < 0.05$) total area under the curve (AUC) and glucose incremental AUC ($P < 0.05$). Additionally, there was a strong negative correlation between fidgeting-induced increases in blood flow and reduced postprandial glucose AUC within the first hour ($r = -0.569$, $P < 0.01$).

Conclusions: Leg fidgeting is a simple, light-intensity physical activity that enhances limb blood flow and can be incorporated during prolonged sitting to improve postprandial glycemic control in people with obesity.

Co-authored publications (5)

J Sleep Res. 2021 Dec;30(6):e13381. doi: 10.1111/jsr.13381. Epub 2021 May 5.

Modest sleep restriction does not influence steps, physical activity intensity or glucose tolerance in obese adults

Jay W Porter [1], Ryan J Pettit-Mee [1], Travis S Emerson [1], Christina S McCrae [2], Guido Lastra [3], Victoria J Vieira-Potter [1], Elizabeth J Parks [1], Jill A Kanaley [1]

Affiliations + expand

PMID: 33949729 DOI: 10.1111/jsr.13381

Abstract

Sleep restriction (SR) (<6 h) and physical activity (PA) are risk factors for obesity, but little work has examined the inter-related influences of both risk factors. In a free-living environment, 13 overweight/obese adults were sleep restricted for five nights to 6 h time-in-bed each night, with and without regular exercise (45 min/65% VO_2 max; counterbalanced design). Two days of recovery sleep followed SR. Subjects were measured during a mixed meal tolerance test (MMT), resting metabolic rate, cognitive testing and fat biopsy (n=8). SR increased peak glucose response (+7.3 mg/dl, p = .04), elevated fasting non-esterified fatty acid (NEFA) concentrations (+0.1 mmol/L, p = .001) and enhanced fat oxidation (p < .001) without modifying step counts or PA intensity. Inclusion of daily exercise increased step count (+4,700 steps/day, p < .001) and decreased the insulin response to a meal (p = .01) but did not prevent the increased peak glucose response or elevated NEFA levels. The weekend recovery period improved fasting glucose (p = .02), insulin (p = .02), NEFA concentrations (p = .001) and HOMA-IR (p < .01) despite reduced steps (p < .01) and increased sedentary time (p < .01). Abdominal adipose tissue (AT) samples, obtained after baseline, SR and exercise, did not differ in lipolytic capacity following SR. Fatty acid synthase protein content tended to increase following SR (p = .07), but not following exercise. In a free-living setting, SR adversely affected circulating NEFAs, fuel oxidation and peak glucose response but did not directly affect glucose tolerance or AT lipolysis. SR-associated metabolic impairments were not mitigated by exercise, yet recovery sleep completely rescued its adverse effects on glucose metabolism.

Keywords: AT; exercise; insulin sensitivity; mixed-meal test; shortened sleep.

› Front Endocrinol (Lausanne). 2020 Sep 2;11:578. doi: 10.3389/fendo.2020.00578. eCollection 2020.

Post Meal Exercise May Lead to Transient Hypoglycemia Irrespective of Glycemic Status in Humans

Jay W Porter [1], Ryan J Pettit-Mee [1], Sean T Ready [1], Ying Liu [1], Guido Lastra [2], Anand Chockalingam [3], Nathan C Winn [4], Laura Clart [1], Jill A Kanaley [1]

Affiliations + expand

PMID: 32982972 PMCID: PMC7492570 DOI: 10.3389/fendo.2020.00578

Free PMC article

Abstract

During exercise, there is coordination between various hormonal systems to ensure glucoregulation. This study examined if hypoglycemia occurs during moderate-intensity exercise in non-obese and obese individuals with and without type 2 diabetes (T2D). Eighteen non-obese, 18 obese, and 10 obese with T2D completed 2 study days that included a meal at 1,800 h followed by rest (NOEX) or exercise (PMEX; 45 min/55% of VO_2 max 2 h post meal). Glucose, insulin, and glucagon concentrations were measured throughout this 5.5 h period. Subjects with T2D had elevated glucose responses to the meal on both study days, compared to non-obese and obese subjects ($P < 0.05$). During evening exercise (PMEX), subjects with T2D had a greater drop in glucose concentration (-98.4 ± 13.3 mg/dL) compared to obese (-44.8 ± 7.1 mg/dL) and non-obese (-39.3 ± 6.1 mg/dL; $P < 0.01$) subjects. Glucose levels decreased more so in females than males in both conditions ($P < 0.01$). Nadir glucose levels <70 mg/dL were observed in 33 subjects during NOEX and 39 subjects during PMEX. Obese males had a larger exercise-induced insulin drop than obese females ($P = 0.01$). During PMEX, peak glucagon concentrations were elevated compared to NOEX ($P < 0.001$). Male participants with T2D had an increased glucagon response during NOEX and PMEX compared to females ($P < 0.01$). In conclusion, in individuals with varying glucose tolerance, there is a dramatic drop in glucose levels during moderate-intensity exercise, despite appropriate insulin concentrations prior to exercise, and glucagon levels rising during exercise. Moderate-intensity exercise can result in low glucose concentrations (<60 mg/dL), and yet many of these individuals will be asymptomatic.

Keywords: exercise; glucagon; glucose; hypoglycemia; type 2 diabetes.

J Appl Physiol (1985). 2020 Aug 1;129(2):283-296. doi: 10.1152/japplphysiol.00174.2020. Epub 2020 Jul 2.

Skeletal muscle microvascular insulin resistance in type 2 diabetes is not improved by eight weeks of regular walking

Lauren K Park [1,2], Elizabeth J Parks [1,3], Ryan J Pettit-Mee [1,2], Makenzie L Woodford [1,2], Thaysa Ghiarone [2], James A Smith [1,2], Allan R K Sales [2,4,5], Luis A Martinez-Lemus [2,6], Camila Manrique-Acevedo [2,7,8], Jaume Padilla [1,2]

Affiliations + expand

PMID: 32614687 PMCID: PMC7473951 DOI: 10.1152/japplphysiol.00174.2020

Free PMC article

Abstract

We aimed to examine whether individuals with type 2 diabetes (T2D) exhibit suppressed leg vascular conductance and skeletal muscle capillary perfusion in response to a hyperinsulinemic-euglycemic clamp and to test whether these two variables are positively correlated. Subsequently, we examined whether T2D-associated skeletal muscle microvascular insulin resistance, as well as overall vascular dysfunction, would be ameliorated by an 8-wk walking intervention (45 min at 60% of heart rate reserve, 5 sessions/week). We report that, relative to healthy subjects, overweight and obese individuals with T2D exhibit depressed insulin-stimulated increases in leg vascular conductance, skeletal muscle capillary perfusion, and Akt phosphorylation. Notably, we found that within individuals with T2D, those with lesser increases in leg vascular conductance in response to insulin exhibited the lowest increases in muscle capillary perfusion, suggesting that limited muscle capillary perfusion may be, in part, linked to the impaired ability of the upstream resistance vessels to dilate in response to insulin. Furthermore, we show that the 8-wk walking intervention, which did not evoke weight loss, was insufficient to ameliorate skeletal muscle microvascular insulin resistance in previously sedentary, overweight/obese subjects with T2D, despite high adherence and tolerance. However, the walking intervention did improve ($P < 0.05$) popliteal artery flow-mediated dilation (+4.52%) and reduced HbA1c (-0.75%). It is possible that physical activity interventions that are longer in duration, engage large muscle groups with recruitment of the maximum number of muscle fibers, and lead to a robust reduction in metabolic risk factors may be required to overhaul microvascular insulin resistance in T2D.**NEW & NOTEWORTHY** This report provides evidence that in sedentary subjects with type 2 diabetes diminished insulin-stimulated increases in leg vascular conductance and ensuing blunted capillary perfusion in skeletal muscle are not restorable by increased walking alone. More innovative physical activity interventions that ultimately result in a robust mitigation of metabolic risk factors may be vital for reestablishing skeletal muscle microvascular insulin sensitivity in type 2 diabetes.

Keywords: blood flow; capillary perfusion; hyperinsulinemia; physical activity; vascular conductance.

› Hypertension. 2019 Dec;74(6):1409-1419. doi: 10.1161/HYPERTENSIONAHA.119.13676. Epub 2019 Oct 21.

Chronic Elevation of Endothelin-1 Alone May Not Be Sufficient to Impair Endothelium-Dependent Relaxation

Zachary I Grunewald [1 2], Thomas J Jurrissen [1 2], Makenzie L Woodford [1 2], Francisco I Ramirez-Perez [2 3], Lauren K Park [1 2], Ryan Pettit-Mee [1 2], Thaysa Ghiarone [1 2], Scott M Brown [4 5], Mariana Morales-Quinones [1 2], James R Ball [1], Kevin F Staveley-O'Carroll [6], Annayya R Aroor [5], Paul J Fadel [7], Pierre Paradis [8], Ernesto L Schiffrin [8 9], Shawn B Bender [1 2 4 5], Luis A Martinez-Lemus [1 2 10], Jaume Padilla [1 2]

Affiliations + expand

PMID: 31630572 PMCID: PMC6854321 DOI: 10.1161/HYPERTENSIONAHA.119.13676

Free PMC article

Abstract

Endothelin-1 (ET-1) is a powerful vasoconstrictor peptide considered to be causally implicated in hypertension and the development of cardiovascular disease. Increased ET-1 is commonly associated with reduced NO bioavailability and impaired vascular function; however, whether chronic elevation of ET-1 directly impairs endothelium-dependent relaxation (EDR) remains elusive. Herein, we report that (1) prolonged ET-1 exposure (ie, 48 hours) of naive mouse aortas or cultured endothelial cells did not impair EDR or reduce eNOS (endothelial NO synthase) activity, respectively ($P>0.05$); (2) mice with endothelial cell-specific ET-1 overexpression did not exhibit impaired EDR or reduced eNOS activity ($P>0.05$); (3) chronic (8 weeks) pharmacological blockade of ET-1 receptors in obese/hyperlipidemic mice did not improve aortic EDR or increase eNOS activity ($P>0.05$); and (4) vascular and plasma ET-1 did not inversely correlate with EDR in resistance arteries isolated from human subjects with a wide range of ET-1 levels (r=0.0037 and r=-0.1258, respectively). Furthermore, we report that prolonged ET-1 exposure downregulated vascular UCP-1 (uncoupling protein-1; $P<0.05$), which may contribute to the preservation of EDR in conditions characterized by hyperendothelinemia. Collectively, our findings demonstrate that chronic elevation of ET-1 alone may not be sufficient to impair EDR.

Keywords: NO synthase; aorta; blood pressure; humans; hypertension.

Randomized Controlled Trial > Med Sci Sports Exerc. 2019 May;51(5):995-1005.
doi: 10.1249/MSS.0000000000001892.

Metabolic Implications of Diet and Energy Intake during Physical Inactivity

Nathan C Winn [1], Ryan Pettit-Mee [1], Lauren K Walsh [1], Robert M Restaino [2], Sean T Ready [1], Jaume Padilla [1 3 4], Jill A Kanaley [1]

Affiliations + expand

PMID: 30694977 PMCID: PMC6465093 DOI: 10.1249/MSS.0000000000001892
Free PMC article

Abstract

Purpose: Physical inactivity is associated with disruptions in glucose metabolism and energy balance, whereas energy restriction may blunt these adverse manifestations. During hypocaloric feeding, higher-protein intake maintains lean mass which is an important component of metabolic health. This study determined whether mild energy restriction preserves glycemic control during physical inactivity and whether this preservation is more effectively achieved with a higher-protein diet.

Methods: Ten adults (24 ± 1 yr) consumed a control (64% carbohydrate, 20% fat, 16% protein) and higher-protein diet (50% carbohydrate, 20% fat, 30% protein) during two 10-d inactivity periods (>10,000 → ~5000 steps per day) in a randomized crossover design. Energy intake was decreased by ~400 kcal·d to account for reduced energy expenditure associated with inactivity. A subset of subjects (n = 5) completed 10 d of inactivity while consuming 35% excess of their basal energy requirements, which served as a positive control condition (overfeeding+inactivity).

Results: Daily steps were decreased from 12,154 ± 308 to 4275 ± 269 steps per day ($P < 0.05$) which was accompanied by reduced $\dot{V}O_2max$ (-1.8 ± 0.7 mL·kg·min, $P < 0.05$), independent of diet conditions. No disruptions in fasting or postprandial glucose, insulin, and nonesterified fatty acids in response to 75 g of oral glucose were observed after inactivity for both diet conditions ($P > 0.05$). Overfeeding+inactivity increased body weight, body fat, homeostasis model assessment of insulin resistance, and 2-h postprandial glucose and insulin concentrations ($P < 0.05$), despite no changes in lipid concentrations.

Conclusions: We show that independent of diet (normal vs higher-protein), mild energy restriction preserves metabolic function during short-term inactivity in healthy subjects. That is, metabolic deterioration with inactivity only manifests in the setting of energy surplus.

Trial registration: ClinicalTrials.gov NCT03013764.

APPENDIX IV – CURRICULUM VITAE

RYAN PETTIT-MEE

EDUCATION

PhD **Exercise Physiology** Expected January 2022
University of Missouri, MO.
Dissertation: "The role of heat shock protein in endothelial insulin resistance: Using passive heat therapy to improve endothelial insulin responsiveness."
Committee: Jill Kanaley PhD (chair, co-mentor), Jaume Padilla PhD (co-mentor), Scott Rector PhD, Luis Martinez-Lemus PhD, DVT, Camila Manrique MD.

MSA **Health Administration** May 2016
Central Michigan University, MI.

MA **Exercise Sciences** August 2014
Central Michigan University, MI.

BSc **Sport and Exercise Science** May 2012
University of Limerick, Ireland

HONORS AND AWARDS

Ben R. Londeree/Tom Thomas Award 2021
University of Missouri, MO.

Edward O'Brien Scholarship 2020
University of Missouri, MO.

Adeline M. Hoffman Graduate Student Fellowship Award 2016-2021
University of Missouri, MO

Michael L. Pollock Student Scholarship 2015
American College of Sports Medicine

Graduate Student Research & Creative Endeavors Grant 2013
Central Michigan University, MI.

PROFESSIONAL EXPERIENCE

Graduate Research and Teaching Assistant, University of Missouri, MO. 2016-Present
Research Experience:

- Dissertation project examining the impact of 7-days of passive heating to endothelial insulin responsiveness and subsequently postprandial blood flow in patients with type 2 diabetes. The project is also evaluating whether heat shock protein 72, a cytoprotective chaperone molecule induced by heat treatment, mitigates JNK activity to improve endothelial insulin signaling

- Led project demonstrating that low-intensity physical activity, in the form of leg fidgeting, performed during prolonged sitting can lower postprandial glucose excursion in people with obesity.
- Assisting with NIH R01 funded project (5R01HL137769-04; PI: Jaume Padilla), examining whether increases in shear stress via increased blood flow (using exercise or passive heating as the stimulus) improves insulin-stimulated blood flow in patients with type 2 diabetes.
- Assisting with NIH R01 funded project (5R01DK101513-05; PI: Jill Kanaley), assessing whether exercise sessions at different times of the day impacts the dawn phenomena (rise in blood glucose before waking) in patients with pre-diabetes or type 2 diabetes.
- Assisted with other graduate student-led projects assessing the impact of physical inactivity on vascular function and sleep restriction and exercise on fat tissue metabolism.

Teaching Experience: Assisted with introductory undergraduate classes to health and fitness, and nutritional topics. Assisted with lecture preparations, exam questions, grading, and exam proctoring. Held course-work review sessions and presented course-work lectures.
Supervisory Experience: Supervised graduate and undergraduate students involved with human and cell culture studies.

Research laboratory Technician, Central Michigan University, MI. 2014-2016
Research Experience: Assisted in projects examining: The insulin sensitizing effects of acute and habitual exercise in insulin resistant participants (PI: Rachel Nelson, PhD); Role of heat shock proteins on inflammatory markers and immune function (PI: Micah Zuhl, PhD); Calorie restriction and exercise interventions on glucose uptake and glycemic control in rodents (PI: Naveen Sharma, PhD).
Supervisory Experience: Supervised graduate and undergraduate students involved in human, animal, and cell c0ulture studies within the laboratory.
General experience: Coordinated research projects, equipment, supplies, and reagents for four start-up principal investigators.

Preventative Cardiology Internship, Henry Ford Health Care System, MI.
2014
Clinical exercise physiology internship in cardiac rehabilitation under guidance of Steven Keteyian, PhD, Jonathan Ehrman, PhD, and Dennis Kerrigan, PhD.

Graduate Research and Teaching Assistant, Central Michigan University, MI 2012 - 2014
Research Experience: Evaluated biomarkers of endothelial dysfunction in insulin resistant participants. Assisted in projects examining: The neural system within exercise-associated muscle cramping (PI: Jeff Edward, PhD), and Insulin sensitizing effects of acute and habitual exercise in insulin resistant participants (PI: Rachel Nelson, PhD).
Teaching Experience: Taught lab component of undergraduate biomechanics course.

RESEARCH SKILLS AND TECHNIQUES

General: Creating and implementing research proposals, research design, data organization, data interpretation, and analytical analysis using Microsoft software programs and statistical software (SPSS, GraphPad); Power calculations (GPower); institutional review board applications and continuing reviews; presenting findings.

Human subject experimental techniques: Duplex doppler ultrasonography; flow-mediated dilation tests; pulse-wave velocity analysis; venous endothelial cell collection and processing;

assisting with skeletal muscle and fat biopsies; assisting hyperinsulinemic-euglycemic clamp procedures; monitoring core temperature using telemetry sensors; indirect calorimetry; maximal graded exercise testing; Dual-energy X-ray absorptiometry; underwater weighing; electrically induced muscle stimulation; blood draws; physical activity and sleep monitoring using accelerometers.

Cell culture experimental techniques: Isolation, passaging, and counting; Loss of function experiments using pharmacological inhibitors and siRNA knockdown.

Rodent experimental techniques: Weighing, feeding, exercising protocols using treadmills and swimming protocols, blood collection, blood pressure measurements, intraperitoneal injections with either saline or insulin stimulation, surgical termination, and dissection.

Biological sample processing and analysis techniques: collecting and processing blood plasma and blood serum samples; tissue homogenization; glucose uptake by rodent tissue using radioactive isotopes; performing assays; using ELISAs; using multiplex kits with Luminex machine; immunofluorescence imaging of endothelial cells; immunoprecipitation; western blotting, mass spectrometry.

PUBLICATIONS

Journal Publications

First Author (1):

Pettit-Mee RJ, Ready ST, Padilla J, Kanaley JA. Leg Fidgeting During Prolonged Sitting Improves Postprandial Glycemic Control in People with Obesity. Obesity (Silver Spring). 2021 Jul;29(7):1146-1154.

Co-authored (9):

Porter JW, **Pettit-Mee RJ**, Emerson TS, McCrae CS, Lastra G, Vieira-Potter VJ, Parks EJ, Kanaley JA. Modest sleep restriction does not influence steps, physical activity intensity or glucose tolerance in obese adults. J Sleep Res. 2021 Dec;30(6):e13381.

Porter JW, **Pettit-Mee RJ**, Ready ST, Liu Y, Lastra G, Chockalingam A, Winn NC, Clart L, Kanaley JA. Post Meal Exercise May Lead to Transient Hypoglycemia Irrespective of Glycemic Status in Humans. Front Endocrinol (Lausanne). 2020 Sep 2;11:578.

Park LK, Parks EJ, **Pettit-Mee RJ**, Woodford ML, Ghiarone T, Smith JA, Sales ARK, Martinez-Lemus LA, Manrique-Acevedo C, Padilla J. Skeletal muscle microvascular insulin resistance in type 2 diabetes is not improved by eight weeks of regular walking. J Appl Physiol (1985). 2020 Aug 1;129(2):283-296.

Winn NC, **Pettit-Mee RJ**, Walsh LK, Restaino RM, Ready ST, Padilla J, Kanaley JA.. Metabolic Implications of Diet and Energy Intake during Physical Inactivity. Medicine and science in sports and exercise. 2019;51(5):995-1005.

Roth J, Szczygiel T, Moore M, O'Connor P, Edwards J, Sharma N, **Pettit-Mee RJ**, Zuhl M.. Profiling Inflammatory Markers during the Competitive Season and Post Season in Collegiate Wrestlers. Journal of Strength and Conditioning Research. 2019;33(8):2153-61.

Grunewald ZI, Jurrissen TJ, Woodford ML Ramirez-Perez FI, Park LK, **Pettit-Mee RJ**, Ghiarone T, Brown SM, Morales-Quinones M, Ball JR, Staveley-O'Carroll KF, Aroor AR, Fadel PJ, Paradis P, Schiffrin EL, Bender SB, Martinez-Lemus LA, Padilla J. Chronic Elevation of Endothelin-1 Alone May Not Be Sufficient to Impair Endothelium-Dependent Relaxation. Hypertension. 2019;74(6):1409-19.

Bourbeau KC, Rosinski MM, Szczygiel TM, **Pettit-Mee RJ**, Sessions JE, Zuhl MN. The stress response in human peripheral mononuclear cells is related to aerobic fitness and Body Mass Index. Gazz Med Ital Arch S. 2019;178(6):417-23.

O'Neil S, Thomas A, **Pettit-Mee RJ,** Pelletier K, Moore M, Thompson J, Barton C, Nelson R, Zuhl M. Exercise Prescription Techniques in Cardiac Rehabilitation Centers in Midwest States. Journal of Clinical Exercise Physiology. 2018;7(1):8-14.

Panza G, Stadler J, Murray D, Lerma N, Barrett T, **Pettit-Mee RJ**, Edwards JE. Acute Passive Static Stretching and Cramp Threshold Frequency. J Athl Train. 2017 Oct;52(10):918-924.

PRESENTATIONS

Pettit-Mee RJ. *Leg fidgeting during prolonged sitting improves postprandial glycemic control in people with obesity*. Oral presentation at the Dept. of Nutrition and Exercise Physiology Seminar Series (October 2020), University of Missouri.

Pettit-Mee RJ, Ready ST, Padilla J, Kanaley JA. *Leg fidgeting during prolonged sitting improves postprandial glycemic control in people with obesity – A pilot study*. Poster presentation at the Annual Meeting of Experimental Biology (April 2019), Orlando, Florida.

Pettit-Mee RJ, Cunningham RP, & Sharma N. *High Casein Diet Differentially Alters FGF21 Levels in Plasma and Cardiac Tissue in Rats.* Poster presentation at the Annual Meeting of Experimental Biology (April 2017), Chicago, Illinois.

Pettit-Mee RJ, Zuhl M, & Sharma N. *The Effect of Calorie Restriction on the Association Between Heat Shock Protein and Akt*. Oral presentation at the 3rd Annual Meeting of the Michigan Physiological (May 2016), Detroit, Michigan.

Pettit-Mee RJ, Horowitz JF, & Nelson RK. *The influence of Exercise and Insulin Resistance on Biomarkers of Endothelial Dysfunction*.
Poster presentation at the 62nd Annual Meeting and 6th World Congress on Exercise is Medicine of the American College of Sports Medicine (June 2015), San Diego, California.
Poster presentation at Central Michigan University Student Research and Creative Endeavors Exhibition (May 2014), Mount Pleasant, Michigan.
Oral presentation at the annual meeting of the Michigan American College of Sports Medicine (February 2014), Gaylord, Michigan.

PROFESSIONAL CERTIFICATIONS

Basic Life Support (CPR and AED), American Heart Association 2021- Present

Clinical Exercise Physiologist, American College of Sports Medicine 2014-Present

PROFESSIONAL AFFILIATIONS

American Physiological Society	2017-Present
American Nutritional Society	2017-Present
American College of Sports Medicine	2014-Present

UNIVERSITY SERVICE

Treasurer, Nutrition and Exercise Physiology Graduate Student Association, 2016-2018
Obtained funding for travel reimbursement to conferences for graduate students.

VITA

Ryan Pettit-Mee was born on February 13th 1990, on the Isle of Wight in the United Kingdom. He earned his undergraduate degree in Sports and Exercise Science at the University of Limerick in Ireland. Ryan then attended Central Michigan University (CMU), where he earned two master's degrees: one in Exercise Science and the other in Health Administration. During his time at CMU, Ryan interned at Henry Ford Cardiac Rehabilitation in Detroit, MI, and became the laboratory research technician of the Exercise Science division. Through his experiences at CMU, Ryan became very interested in understanding more about the relationship between endothelial insulin resistance contributing to cardiovascular disease in type 2 diabetes. Subsequently, Ryan pursued a doctorate at the University of Missouri under the mentorship of Dr. Jaume Padilla for his work on exercise, physical activity, and endothelial insulin resistance. Shortly after enrolling at Mizzou, Ryan became co-mentored by Dr. Jill Kanaley and expanded his training into research areas involving exercise and the dawn phenomenon and metabolic implications associated with disrupted sleep. Ryan earned his doctorate in Exercise Physiology in January 2022. Ryan accepted a post-doctoral position at Wake Forest in North Carolina, examining the interplay between sleep patterns, type 2 diabetes, cerebral vasculature, and Alzheimer's disease. Ryan will assess the use of interventions to improve insulin sensitivity, such as exercise, and assess their impact on this convoluted interplay.