IMPACT OF DIETARY SUPPLEMENTATION OF GLYCOCALYX PRECURSORS ON VASCULAR FUNCTION IN TYPE 2 DIABETES

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by
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IMPACT OF DIETARY SUPPLEMENTATION OF GLYCOCALYX PRECURSORS
ON VASCULAR FUNCTION IN TYPE 2 DIABETES

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DEDICATION

I would like to dedicate this dissertation to my father, James Smith. Thank you for your sacrifices over the years and especially during my childhood to allow me the opportunities to attend higher education. Your commitment and dedication provided the foundation necessary for me to achieve my goals and to have a life worth looking forward to. Without you, I would not be where I am today.

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LIST OF ABBREVIATIONS

T2D: type 2 diabetes

DSGP: dietary supplementation of glycocalyx precursors

CVD: cardiovascular disease

NO: nitric oxide

PI3K: phosphatidylinositol 3-kinase

IRS-1/2: insulin receptor substrate 1/2

RAS: renin-angiotensin system

MAPK: mitogen-activated protein kinase

ET-1: endothelin-1

eNOS: endothelial nitric oxide synthase

VSMC: vascular smooth muscle cells

GLUT4: glucose transporter 4

cGMP: cyclic guanosine monophosphate

L-NMMA: NG-monomethyl-L-arginine

PIP2: phosphatidylinositol 4,5-bisphosphate

IP3: inositol 1,4,5-trisphosphate

MLCK: myosin light chain kinase

CO_2: carbon dioxide

FMD: flow-mediated dilation

HOMA-IR: homeostasis model assessment of insulin resistance

AGEs: advanced glycation end products

VCAM-1: vascular cell adhesion molecule

LDL: low-density lipoproteins

RUN: chronic voluntary wheel running

CR: caloric resistriction

L-NAME: NG-nitro-l-arginine methyl ester

CD44: hyaluronic acid receptor

PBR: perfused boundary region

Ach: acetylcholine

SNP: sodium nitroprusside

VA: veterans affairs

HUVECs: human umbilical vein endothelial cells

ABPM: ambulatory blood pressure monitor

cfPWV: carotid-femoral pulse wave velocity

TPR: total peripheral resistance

SEM: standard error of the mean

WGA: wheat germ agglutinin

MAP: mean arterial pressure

SkM: skeletal muscle

Einc: incremental modulus of elasticity

AUC: area under the curve

SGLT2: sodium-glucose cotransporter-2

GLP-1: glucagon-like peptide-1

FFA: free fatty acids

ABSTRACT

Degradation of the endothelial glycocalyx in type 2 diabetes (T2D) is thought to contribute to impaired shear stress mechanotransduction, leading to endothelial dysfunction and the development of cardiovascular disease. Herein, it was hypothesized that restoration of the endothelial glycocalyx with dietary supplementation of glycocalyx precursors (DSGP, containing glucosamine sulfate, fucoidan, superoxide dismutase, and high molecular weight hyaluronan) improves endothelial function and other indices of vascular function in T2D. First, in db/db mice, treatment with DSGP (100 mg/kg/day) for four weeks restored endothelial glycocalyx length, as assessed via atomic force microscopy in aortic explants. Restoration of the glycocalyx with DSGP was accompanied by improved flow-mediated dilation (FMD) and reduced arterial stiffness in isolated mesenteric arteries. Further corroborating these findings, treatment of cultured endothelial cells with that same mixture of glycocalyx precursors promoted glycocalyx growth. Next, as an initial step to investigate the translatability of these findings, a pilot (n=22) double-blinded randomized placebo-controlled clinical trial was conducted to assess the effects of DSGP (3,712.5 mg/day) for eight weeks on endothelial glycocalyx integrity and indices of vascular function, including FMD, in Veterans with T2D. Contrary to the hypothesis, DSGP neither enhanced endothelial glycocalyx integrity nor improved vascular function indices relative to placebo. Together, these findings conceptually support the notion that restoration of the endothelial glycocalyx can lead to improvements in vascular function in a mouse model of T2D; however, DSGP as a therapeutic strategy to

enhance vascular function in individuals with T2D does not appear to be efficacious.

CHAPTER 1: BACKGROUND

Vascular complications of Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2D) prevalence is rapidly rising globally and within the United States (1) with one in three individuals projected to be diagnosed with T2D by 2050 (2). In particular, T2D is widespread among the Veteran population, and the Veterans Affairs (VA) administration spends $1.5 billion annually on diabetes care (3, 4). Importantly, T2D contributes to the staggering rates of cardiovascular disease (CVD) and cardiovascular mortality in this population (5). A characteristic feature of T2D is insulin resistance, or the blunted ability of peripheral tissues to regulate glucose homeostasis in response to insulin (6). In addition to the metabolic complications of T2D are the increased CVD risk and complications associated with T2D resulting in a decreased quality of life (7). Under normal conditions, insulin promotes nitric oxide (NO)-dependent increases in insulin and glucose delivery to skeletal muscle via stimulation of skeletal muscle resistance artery vasodilation and increased skeletal muscle capillary perfusion (8–10), which accounts for up to 40% of skeletal muscle glucose uptake postprandially (11). However, in the presence of insulin resistance, commonly found in individuals with obesity and T2D, selective impairment of insulin-stimulated vasodilation is observed, causing endothelial dysfunction and inflammation, which is associated with attenuated skeletal muscle glucose uptake, nutrient delivery, and dysglycemia. Previous research indicates that vascular insulin resistance is an early contributor to the development of metabolic disease and precedes that of whole-body insulin resistance (12, 13). Indeed, individuals with T2D have selective impairment of insulin-stimulated vasodilation, that is, reductions in insulin receptor substrate 1/2 (IRS-1/2)/phosphatidylinositol 3-kinase (PI3K) and NO signaling with maintained or elevated Renin-angiotensin system/Mitogen-activated protein kinase/ Endothelin-1 (RAS/MAPK/ET-1) signaling (8, 14–19). Lifestyle modifications, such as increased exercise participation, are recommended as a first-line therapy to manage T2D and decrease CVD risk (20), however, evidence suggests that exercise training in patients with T2D does not evoke optimal cardiometabolic (21–24) or cardiorespiratory fitness (25) adaptations as expected. Such restrictions warrant new adjuvant therapies targeting the vasculature and a better understanding of the mechanisms responsible for such limitations. This dissertation seeks to identify a novel therapy for patients with T2D with the goal of improving insulin signaling and overall vascular health.

Vascular endothelial cell signaling in health and insulin resistance

In addition to the critical role that insulin plays in regulating glucose homeostasis, insulin signaling in the vasculature plays a pivotal, albeit lesser-known role in promoting vascular health. In health, insulin binds to an insulin receptor located throughout the arterial tree to promote increased insulin and glucose delivery to skeletal muscle via skeletal muscle vasodilation and augmented capillary perfusion (8–10). This occurs due to IRS stimulation that leads to PI3K activation with subsequent phosphorylation of endothelial nitric oxide synthase (eNOS) (26) and eventual NO production (27). NO will diffuse to the underlying vascular smooth muscle cells (VSMC) to ultimately trigger vasodilation of the endothelium (14, 15, 28). Activation of the IRS/PI3K/eNOS signaling pathway possesses an anti-atherogenic and anti-inflammatory milieu that is critical for maintenance of vascular health and function via NO-dependent mechanisms (18, 29). Concurrently, insulin also stimulates the RAS/MAPK/ET-1 signaling pathway which has been linked to inflammatory and atherogenic development (30). In health the net result of these two opposing signaling cascades is vasodilation, however in insulin resistance, commonly found in individuals with obesity and T2D, selective impairment of the insulin receptor causes reductions in IRS/PI3K/NO signaling and maintained or elevated RAS/MAPK/ET-1 signaling (8, 14–19). The loss of protective insulin signaling in endothelial cells results in the development of atherosclerosis (31) and impaired endothelial function (19, 24, 32–38) compared to healthy controls. This asymmetry between NO and ET-1 production is a cardinal feature of insulin resistance and vascular endothelial dysfunction. Modalities to improve insulin sensitivity in the vasculature are warranted for combatting vascular derangements in insulin resistance.

Exercise as a therapeutic target for improving vasculo-metabolic health

Public health guidelines recommend that adults should engage in 150 to 300 minutes of moderate intensity or 75 minutes of vigorous intensity weekly with moderate-to high-intensity resistance training at least two days per week. Research from animals and humans demonstrate exercise as a potent therapeutic for enhancing capillary perfusion and insulin-stimulated vasodilation with subsequent increases in blood flow to skeletal muscle (29, 39–44), however, in the United States 80% of adults are insufficiently active (45). Acute exercise removal studies have highlighted the severity of exercise cessation (13), such as impairments in whole-body glucose metabolism, weight gain, and declines in vascular insulin sensitivity, thus emphasizing the important of regular exercise sessions. Indeed, immediately after exercise insulin sensitivity and skeletal muscle glucose uptake are augmented for up to 48 hours later (46) due to, but not limited to, increases in glucose transporter 4 (GLUT4), insulin receptor protein content, eNOS, and NO bioavailability (47, 48). Additionally, the improvements noted in the vasculature are, in part, NO-mediated, but also shear stress mediated (49, 50), which are blunted in disease states like T2D (34).

Shear stress as a potent vascular-sensitizing agent

Research from animals and humans show that exercise is effective in enhancing insulin-stimulated vasodilation and skeletal muscle blood flow (29, 39–44). These beneficial responses appear to be restricted to vascular beds that experience increases in blood flow during exercise, independent of changes in body composition or reductions in hyperglycemia (39–41). While the precise mechanisms are unknown evidence suggests that the improved vascular adaptations following exercise are due to shear stress, or the tangential force of blood flow on the endothelial surface in blood vessels (51). This phenomenon has been directly tested in endothelial cells that were subjected to shear stress for three hours compared to non-sheared endothelial cells with sheared cells exhibiting significant increases in eNOS expression that remained elevated for 12 hours post-flow exposure (52). This observation has been recapitulated in isolated perfused arteries exposed to high flow versus no flow conditions with enhanced eNOS expression, NO production, and improved endothelium-dependent vasodilation noted (53).

To corroborate these preclinical findings in humans, one-legged exercise for 60 minutes was implemented in healthy humans with the rested contralateral leg used as a control (39). Results from this experimental design revealed that the exercised leg improved insulin-stimulated vasodilation and skeletal muscle microvascular perfusion compared to the control leg. Importantly, the beneficial impact of shear stress on improving the responsiveness of the vasculature occurs independent of exercise, as demonstrated in several studies utilizing heat as a stimulus for increasing blood flow with one arm left intact and the other arm occluded with a blood pressure cuff to limit increases in blood flow (i.e., shear stress). Only favorable adaptations were observed in the non-occluded arm compared to the occluded arm, highlighting the necessity for increased blood flow-induced shear stress to exert beneficial vascular responses (54–56). Taken together, shear stress not only renders the endothelium more insulin responsive, but it also amplifies insulin signaling and subsequent insulin-stimulated vasodilation in scenarios that include exercise (29, 39, 49) and no exercise (e.g., heating) (50). These results are typically associated with significant increases in microvascular perfusion (50, 57) and skeletal muscle glucose uptake (46, 58, 59), depending on the severity of dysfunction.

Restoring the endothelial glycocalyx to combat vascular derangements in T2D

A proposed explanation responsible for the exercise deficits observed in T2D is impaired vascular endothelial glycocalyx integrity (i.e., length) (60). The endothelial glycocalyx, located within the apical surface of the endothelium, is a functional, hair-like structure that detects blood flow-induced shear stress throughout the arterial tree and converts shear forces into signals that provide messages to the underlying VSMC including vasoconstriction, vasodilation, remodeling, and inflammation (61). Previous reports have indicated that a thick glycocalyx represents a healthy, vascular protective phenotype while an impaired, or thin (i.e., reduced length), glycocalyx represents a damaged phenotype, predisposing the vascular endothelium to endothelial dysfunction and cardiovascular disease (60–64). While decreased glycocalyx length has been positively correlated with blunted endothelial function, including impaired eNOS synthesis and NO production (65), glycocalyx-denuded cells and blood vessels have also demonstrated shear-induced NO production is absent (66). Therapeutic interventions to restore the length of the glycocalyx are underway in clinical trials but are limited. Recently, the nutritional supplement, Endocalyx®, was developed with the aim to protect and restore the endothelial glycocalyx (67). This supplement contains several dietary glycocalyx precursors (hyaluronic acid, glucosamine, antioxidants, and fucoidan) to restore the overall glycocalyx structure (67). Previous evidence in mice have shown that 10-weeks of dietary Endocalyx® supplementation improved the endothelial glycocalyx, augmented NO content, enhanced endothelium-dependent vasodilation, and reduced aortic stiffness compared to controls (68). In humans, the literature is limited, with recent evidence in older adults indicating that three months of Endocalyx® supplementation was not capable of restoring overall endothelial function or glycocalyx integrity compared to placebo (69). In South-Asian Surinamese descent individuals with T2D (n=19), three months of Endocalyx® supplementation improved inflammatory markers and microvascular endothelial health compared to a diet intervention (70), warranting larger clinical trials to investigate the feasibility of Endocalyx® as a therapeutic target to ameliorate vasculo-metabolic derangements and improving health outcomes in T2D. Accordingly, the overarching hypothesis of this dissertation was to determine if supplementation with dietary supplementation of glycocalyx precursors (DSGP) in a pilot, randomized, double-blind, placebo-controlled eight-week clinical trial would ameliorate vascular dysfunction in Veterans present with T2D. The specific aims of this dissertation are as follows:

Specific aim 1: To document that DSGP rectifies endothelial glycocalyx integrity in Veterans with T2D.

Hypothesis: Veterans with T2D who receive DSGP will exhibit enhanced glycocalyx integrity compared to Veterans with T2D who receive placebo pills.

Specific aim 2: To demonstrate the critical role of the endothelial glycocalyx in overall vascular function in Veterans with T2D.

Hypothesis: An impaired endothelial glycocalyx attenuates overall vascular function in Veterans with T2D.

CHAPTER 2: EXTENDED LITERATURE REVIEW

Insulin resistance as a problem

In 2021, it was estimated that 38.4 million people, or 11.6% of the population in the United States was diagnosed with diabetes (1). The national financial burden of diabetes is estimated at $412.9 billion as individuals diagnosed with diabetes, on average, have medical expenditures 2.6 times higher than those without diabetes (71). Future projections have estimated that one in three individuals in the United States will be diagnosed with T2D, a disease characterized by skeletal muscle insulin resistance and hyperglycemia by 2050 (2, 72). Alongside the metabolic complications of T2D are the increased CVD risk and complications associated with T2D resulting in a decreased quality of life (7). Indeed, adults with diabetes have a 50% higher risk of all-cause mortality compared to normoglycemic adults (73). Lifestyle interventions are effective in preventing or delaying the onset of T2D, however, excess calorie consumption that develops into obesity (74), and physical inactivity, continues to contribute to the annual increase and development of T2D and plays a causal role in the impaired insulin signaling pathway and endothelial dysfunction (75). While the complications of T2D and insulin resistance are multifaceted and involve multiple mechanistic impairments throughout the body, this dissertation literature review will focus on insulin sensitivity in the vasculature, endothelial (i.e. vascular) function, glycocalyx structure and function, and adjuvant therapeutic strategies to combat vascular dysfunction and restore the endothelial glycocalyx.

Systemic insulin resistance in T2D

The development of T2D is progressive and stems from the combination of genetic and environmental factors. Evidence suggests that mothers with diabetes or who are obese during pregnancy give birth to offspring that have insulin resistance, or the blunted ability of peripheral tissues to regulate glucose homeostasis in response to insulin (6). Additionally, these offspring are at an increased risk of developing diabetes later in life, thus indicating some genetic factors in the development of T2D (76). Environmental factors, such as physical inactivity and obesity, also play a primary role in the development of T2D (74, 77). Cumulatively, these factors work synergistically in the development of insulin resistance that can subsequently result in T2D if proper treatment is not implemented, thus highlighting the need for better management and prevention of T2D.

In a metabolically healthy individual (i.e., normoglycemic), ingestion of a meal results in insulin release from the pancreatic beta cells with subsequent suppression of glucose output from the liver to promote skeletal muscle glucose uptake. These metabolic actions result in steady control of blood glucose concentrations. However, due to a combination of genetic and environmental factors, normoglycemic individuals can progress to an insulin resistant state known as prediabetes (78). Prediabetic individuals demonstrate a normal or slightly elevated blood glucose response following a meal, otherwise known as impaired glucose tolerance, which is offset by a large rise in plasma insulin secretion. If these individuals are left untreated, they may progress to a complete insulin resistant state, otherwise known as T2D. Individuals with T2D demonstrate increased blood glucose concentrations following a meal which is not offset by an appropriate plasma insulin response. This is due to failure of the pancreatic beta cells being able to adequately release insulin into the plasma to counteract the hyperglycemic response following a meal (79), as well as, impaired systemic insulin signaling resulting in the decreased capacity of glucose to be utilized within the body. These cumulative impairments result in the attenuation of skeletal muscle to remove glucose from the bloodstream and thus the inability to regulate postprandial glycemia.

While much is known about the role of skeletal muscle insulin resistance in the progression of T2D, the role of the vasculature in the development of T2D is not as well understood. In normal healthy states, insulin promotes increased insulin and glucose delivery to skeletal muscle via stimulation of skeletal muscle resistance artery vasodilation and increased skeletal muscle capillary perfusion (8–10). In settings of insulin resistance, however, the cardiovascular actions of insulin can become impaired and cause skeletal muscle resistance arteries to decrease skeletal muscle glucose uptake, contributing to the impaired glucose homeostasis (38, 80–82). Further evidence shows impaired insulin signaling in endothelial cells of large conduit arteries increases atherosclerotic disease risk (31, 83), thus emphasizing the potential role of the vascular endothelium as an early contributor to the manifestation of whole-body insulin resistance. To substantiate this notion, previous work in rodent models indicate that impairments in vascular insulin signaling precede the detection of insulin resistance in metabolically active tissue, such as skeletal muscle (12, 36). Interestingly, these results have been translated in healthy, young, recreationally active men who underwent a short-term (e.g., 10-day) obesogenic lifestyle, characterized by decreased ambulation and increased calorie consumption, that indeed, vascular insulin resistance precedes the development of whole-body insulin resistance (13). To date, this was the first piece of evidence in humans to show this phenomenon and it is not known if these results would persist in an older or dysglycemic cohort, such as T2D.

Vascular endothelial cell signaling

Vascular endothelial cells contain an insulin receptor throughout the arterial tree. In normal, healthy conditions (i.e., normoglycemia), insulin will bind to an insulin receptor located on the surface of endothelial cells to trigger a signaling cascade that ultimately results in vasodilation due to the production and release of nitric oxide (NO). To elucidate, insulin binds to an insulin receptor on the endothelial cell surface to stimulate IRS-1/2/PI3K signaling, resulting in the phosphorylation of eNOS at serine 1177 (26). eNOS then catalyzes the conversion of L-arginine and O_2 to L-citrulline and NO (27). L-citrulline eventually enters the circulation for reuptake in the kidneys to get recycled into L-arginine to later be converted into nitric oxide (84). Subsequently, NO will diffuse to the underlying VSMC to stimulate guanyl cyclase, the enzyme responsible for the formation of cyclic guanosine monophosphate (cGMP) (28). Production of cGMP results in vasodilation of the VSMC and ultimately causes vasodilation of the endothelium (14, 15, 28). Activation of the IRS/PI3K/eNOS signaling pathway possesses an anti-atherogenic and anti-inflammatory milieu through its production and bioavailability of NO, which not only plays an important role in vasodilation, but also insulin-stimulated production of NO as a mediator of vascular health and function (18, 29). The first piece of evidence in humans to demonstrate the physiological effects of insulin in the vasculature was published in 1939 (85). In this study, a bolus subcutaneous injection of insulin augmented peripheral blood flow, establishing the theory that insulin possesses a vasodilatory role. In 1994, Scherrer *et al.* established that the vasodilatory effects of insulin observed in the peripheral vasculature is mediated by NO (86). Briefly, healthy volunteers were infused with NG-monomethyl-L-arginine (L-NMMA), a specific inhibitor of eNOS, both before, and at the end of a two-hour hyperinsulinemic-euglycemic clamp in the brachial arteries to examine the role of NO in insulin-mediated vasodilation in the peripheral vasculature. L-NMMA (but not norepinephrine, an NO-independent vasoconstrictor) caused greater reductions in forearm blood flow during hyperinsulinemia compared to baseline. Furthermore, L-NMMA restricted insulin-induced vasodilation throughout the two-hour clamp, leading to significant increases in arterial pressure. Overall, the major finding from this study exemplified the key role of NO signaling during insulin-stimulated vasodilation.

Concurrently, insulin also stimulates the production of the potent vasoconstrictor ET-1 via the RAS/MAPK signaling pathway (8, 29, 87). Stimulation of ET-1 has been linked to inflammatory and atherogenic development (30). ET-1 is secreted via the endothelial cells and binds to an ET_A

receptor located in the VSMC to cause vasoconstriction through a signaling cascade that increases oxidative stress and promotes VSMC growth and proliferation (88). To elucidate, once ET-1 is bound to ET_A receptors G-proteins will activate various downstream pathways, including the activation of phospholipase C which will hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2). The breakdown of PIP2 will generate inositol 1,4,5-trisphosphate (IP3), a second messenger, that will interact with the endoplasmic reticulum to cause the release of stored calcium ions (Ca^{2+}) into the cytoplasm. The increased cytosolic Ca^{2+} concentration will bind to calmodulin, a calcium binding protein, to activate myosin light chain kinase (MLCK). MLCK subsequently activates myosin light chain that ultimately leads to the contraction of VSMC (89, 90), thus narrowing the lumen and reducing blood flow. Taken together, the observation that insulin applies NO-dependent vasodilatory and ET-1-dependent vasoconstrictive effects have been consistently exhibited in humans (16, 18, 86, 91), various animal models *in vivo* (35), and in isolated arteries *in vitro* (36, 92, 93).

To mechanistically interrogate this phenomenon, Eringa *et al.* probed the roles of NO and ET-1 in acute responses (i.e., 30 minutes) of insulin in isolated rat cremaster skeletal muscle first-order arterioles (94). Results indicated that physiologic concentrations of insulin solely failed to significantly influence arteriolar diameter, however, when ET-1 receptors were inhibited, there were significant increases in insulin-induced vasodilation compared to baseline values. The observed vasodilatory response was subsequently eradicated with the administration of an NO synthesis inhibitor, which then resulted in insulin-induced vasoconstriction. These results provided initial evidence that physiological concentrations of insulin during NO synthesis inhibition were vasoconstrictive, and that this phenomenon was mediated by ET-1. Indeed, similar findings have been reported (18, 36, 95) to show impairments in insulin-stimulated vasodilation. A primary mechanism involved in this pathological observation commonly found in individuals with obesity and insulin resistance is the disproportion in endothelial cell insulin signaling, represented by a reduction in IRS/PI3K/NO signaling with either no change or elevated RAS/MAPK/ET-1 signaling (12, 36, 94, 96), termed "selective insulin resistance" (97, 98).

Insulin resistance impairs endothelial cell signaling vasodilation in the vasculature

Endothelial insulin signaling involves a balance between vasodilator/anti-inflammatory/anti-atherogenic IRS/PI3K/NO and vasoconstrictor/pro-inflammatory/pro-atherogenic RAS/MAPK/ET-1 signaling pathways (Figure 2.1) (29). In normal healthy conditions, the net result of these two opposing signaling cascades is vasodilation. In the presence of insulin resistance, commonly found in individuals with obesity and T2D, selective impairment of insulin-stimulated IRS/PI3K/NO signaling is observed, which consequently results in the reduction of insulin-stimulated NO-mediated vasodilation and maintained or elevated RAS/MAPK/ET-1 signaling (8, 14–19). Indeed, experimental data shows that vascular responses to measures of endothelial function are impaired in obese animal models (35, 36), non-diabetic obese women (37) and men (38), and patients with T2D (19, 24, 32–34) compared to healthy controls. This asymmetry between NO and ET-1 production is a cardinal feature of insulin resistance and vascular endothelial dysfunction.

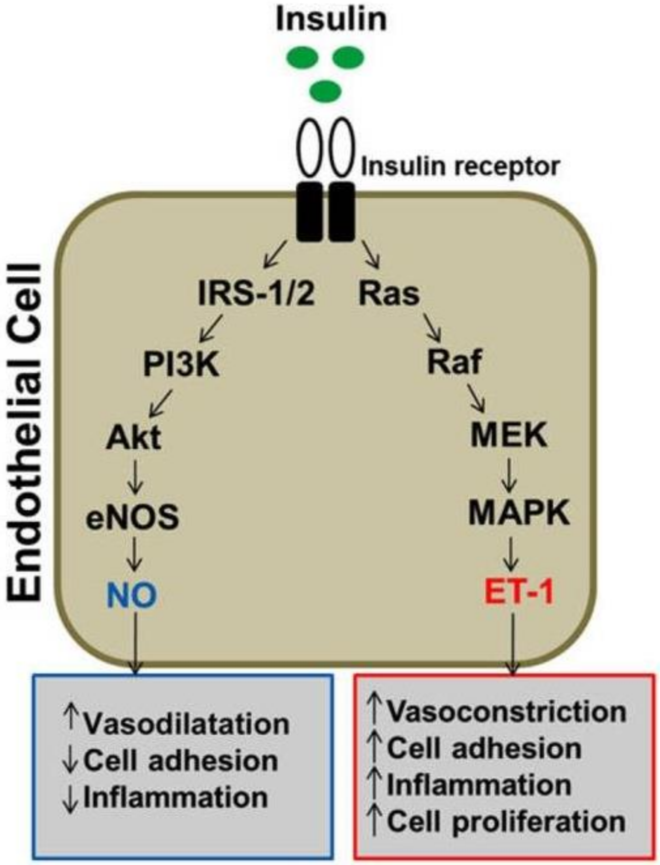

Figure 2.1. Insulin signaling in vascular endothelial cells. Figure adapted from Padilla *et al*. (29).

To expand on this, isolated resistance cremaster muscle arteries taken from obese, insulin resistant Zucker and lean control rats were stimulated with insulin to investigate the roles of NO and ET-1 in healthy versus obese and insulin resistant (36). These arteries were treated with a NO synthesis inhibitor and an ET-1 receptor antagonist followed by exposure to insulin. In arteries of obese rats, insulin produced endothelin-mediated vasoconstriction whereas insulin stimulation in arteries from lean rats increased both NO and ET-1 generation, resulting in a neutral response under basal conditions. When endothelin receptors were blocked in arteries from lean rats, insulin induced vasodilation, but not in obese rats. Moreover, when NO synthesis was inhibited, arteries from lean rats underwent vasoconstriction whereas arteries from obese rats remained unchanged. Taken together, these results provide evidence of selective insulin resistance in an animal model demonstrating that obesity present with insulin resistance, compared to lean insulin-sensitive controls, causes a shift from a protective, vasodilatory phenotype to an inflammatory, vasoconstrictive phenotype due to the reduction in NO signaling coupled with elevated ET-1 signaling (36). These results have been replicated in non-diabetic humans comparing lean versus obese, insulin resistant patients (16). Specifically, lean, healthy, and obese, insulin resistant patients underwent insulin and ET-1_A antagonist (BQ123) infusion to document if increased endogenous endothelin stimulation contributes to skeletal muscle insulin resistance due to impaired insulin-stimulated vasodilation. Results indicated that endothelin antagonism enhanced skeletal muscle leg blood flow in response to insulin in obese, insulin resistant patients while there were no changes in the healthy cohort (16). Similar findings in humans with obesity and T2D versus lean healthy controls have been reported with one-hour insulin infusion blunting leg blood flow in T2D and elevating ET-1 concentrations compared to healthy controls (24).

Insulin-stimulated increases in blood flow are critical for insulin delivery and skeletal muscle glucose uptake

Endothelial insulin signaling plays a crucial role in glycemic control via the microvasculature, or small blood vessels inside the body. The microvasculature is responsible for oxygen and nutrient delivery to tissues and removes carbon dioxide (CO_2) and metabolic waste products from tissues (99). Evidence shows that insulin aids in its own delivery and transport into skeletal muscle for glucose uptake (96, 100). Insulin induces capillary (i.e., microvascular) recruitment and increased microvascular blood flow within skeletal muscle to deliver insulin and glucose to metabolically active tissues. This process occurs within five to 10 minutes and is NO-dependent (57). Upon insulin stimulation, non-perfused capillaries will be recruited to increase the endothelial surface area for nutrient and waste exchange between blood supply and skeletal muscle (96). Additionally, microvascular recruitment decreases vascular resistance, or the difficulty of blood to flow through an area, allowing for increased skeletal muscle perfusion and blood flow through feeding resistance and conduit arteries (96). Increased blood flow through conduit arteries follows later, typically between 30 to 120 minutes (11), depending on several factors such as the concentration and duration of insulin stimulation (101, 102), population (32), and the limb being measured (103).

Previous work from Vincent *et al.* determined the time course extent of skeletal muscle blood flow and capillary recruitment in rats to aid in insulin-stimulated skeletal muscle glucose uptake under physiological infusion of insulin (57). In this study, the time sequence of vascular and metabolic responses to a 30-minute physiological insulin infusion were investigated *in vivo* using rat skeletal muscle. Briefly, three groups of rats were infused with either saline only, insulin only, or insulin combined with a NO synthesis inhibitor. During the 30-minute infusion period, blood flow in the femoral artery and microvascular blood volume in the proximal adductor muscle group were quantified using ultrasonography. It was observed that insulin induced capillary recruitment occurred within five to 10 minutes of infusion and this recruitment preceded activation of insulin-signaling pathways, increases in skeletal muscle glucose uptake, and changes in total leg blood flow. However, when NO synthesis was inhibited, both capillary recruitment and skeletal muscle glucose uptake were prevented, providing evidence that insulin recruits skeletal muscle capillaries *in vivo* by an NO-dependent mechanism and that prevention of capillary recruitment blunts skeletal muscle glucose uptake.

Another piece of evidence to help quantify the vascular actions of insulin was the critical work done by Baron *et al.* in which it was established that insulin stimulated vasodilation and the subsequent increases in blood flow are responsible for up to 40% of skeletal muscle glucose uptake postprandially (11). Briefly, nine lean, insulin-sensitive subjects underwent a 90-minute hyperinsulinemic-euglycemic clamp on two separate occasions that were ~four weeks apart with insulin-stimulated vasodilation left intact or inhibited via intra-femoral artery infusion of L-NMMA to better understand the time course of skeletal muscle glucose uptake and its modulation by changes in perfusion. During the experiment, leg blood flow and arteriovenous glucose difference were recorded every 10 minutes with leg glucose uptake calculated as leg glucose uptake = leg blood flow x arteriovenous glucose difference. It was observed that the systemic insulin infusion caused a time-dependent increase in leg blood flow and that this response was completely abolished with L-NMMA infusion. In summary, results from the previously mentioned studies reaffirms that the vascular actions of insulin are, to some degree, responsible for insulin's overall metabolic activity in skeletal muscle and that insulin-stimulated blood flow is imperative in regulating glucose homeostasis.

Vascular insulin resistance and impaired insulin-stimulated blood flow contributes to glycemic dysregulation

As previously mentioned, insulin-stimulated microvascular recruitment and subsequent blood flow are NO-dependent, with human and animal studies showing inhibition of NO to be deleterious for endothelial insulin responsiveness and subsequent skeletal muscle glucose uptake (11, 35, 57, 82, 104). The reciprocal has also been shown; that is, when ET-1 antagonism is present there are enhancements in insulin-stimulated blood flow and skeletal muscle glucose uptake (16, 105). Evidence dates back to the 1990's in which insulin-stimulated increases in leg blood flow during a hyperinsulinemic-euglycemic clamp were attenuated in obese, non-diabetic (38) and obese, T2D (32) individuals compared to lean, normoglycemic controls. The blunted vascular insulin response that occurs in obesity and T2D, demonstrated via reduced insulin-stimulated leg blood flow during a hyperinsulinemic-euglycemic clamp, contributes to the reductions in skeletal muscle glucose uptake, as assessed via arteriovenous glucose differences, as illustrated in Figure 2.2B. Taken together, these studies provide key pieces of early evidence that insulin-stimulated vasodilation is attenuated in obesity with partial and complete insulin resistance, and that this aberrant response results in the inability of skeletal muscle to uptake glucose and maintain proper glycemic control.

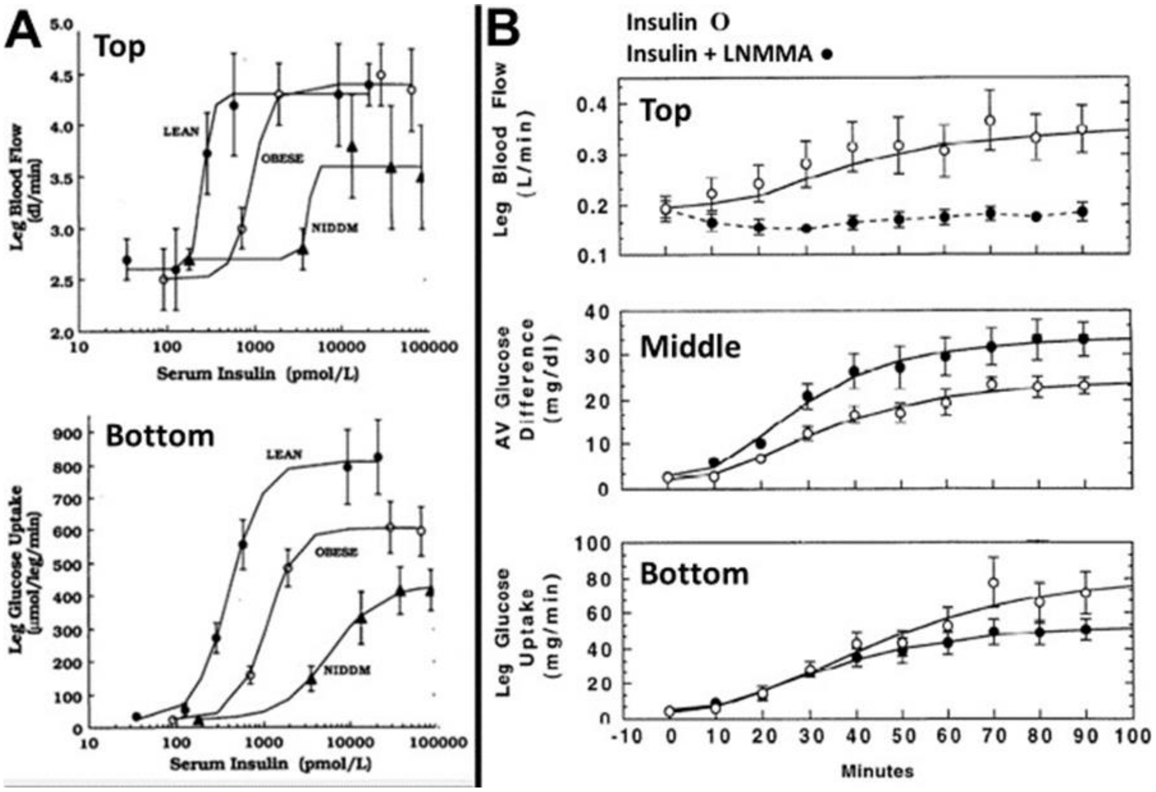

Figure 2.2. Impaired insulin-stimulated leg blood flow reduces skeletal muscle glucose uptake. Panel A shows leg blood flow (top) and skeletal muscle glucose uptake (bottom) during a hyperinsulinemic-euglycemic clamp in lean, healthy (black circles), obese, non-diabetic (white circles), and obese, T2D (NIDDM; black triangles). Adapted from Laakso *et al.* (32). Panel B shows leg blood flow (top), arteriovenous glucose differences (middle), and skeletal muscle glucose uptake (bottom) during a hyperinsulinemic-euglycemic clamp (white circles) or

co-infusion with L-NMMA (black circles) in lean, healthy humans. Adapted from Baron *et al.* (11). Layout of figure adapted from Padilla *et al.* (29).

To better understand the relationship between obesity, insulin resistance, inflammation, endothelial dysfunction, and increased cardiovascular disease risk, Kim *et al.* (12) investigated the time course of which vascular tissue becomes susceptible to the development of inflammation and insulin resistance in mice fed an equicaloric diet of either a control-low fat (10% saturated fat) or a high-fat content (60% saturated fat) for a duration of one to 14 weeks. Following the study intervention one-half of the animals in each study group received either vehicle (normal saline) or insulin injection following an overnight fast. Thoracic aorta, liver, skeletal muscle, and adipose tissue were collected and probed for various proteins. Across the 14-week study period, mice fed a high-fat diet steadily increased their bodyweight and percent body fat with the results becoming statistically significant after four weeks of high-fat feeding. Additionally, and as expected, fasting insulin concentrations remained constant over the 14-weeks in mice fed a low-fat diet whereas fasting insulin concentrations were ~4-fold higher in the mice fed a high-fat diet by the week four timepoint and remained significantly elevated throughout the study, consistent with the development of insulin resistance. Next, circulatory markers of chronic inflammation, a characteristic feature of obesity and involved in the pathogenesis of insulin resistance, were assessed. When comparing the low-fat and high-fat diet groups, circulating markers of chronic inflammation were not significantly elevated until the end (week 14) of the study. Moreso, when examining inflammation and NO content in vascular tissue, it was discovered that vascular inflammation and endothelial dysfunction were observed within one week of the onset of high-fat feeding and that this effect persisted throughout the remaining 14-week study. This was further emphasized via the reduction of NO content and vascular insulin signaling transduction proteins (Akt and phosphorylated eNOS) within just one week of high-fat feeding compared to low-fat fed controls. Thus, vascular inflammation and endothelial dysfunction are early indications of diet-induced obesity and insulin resistance and precede the development of elevated circulatory inflammatory markers.

Interestingly, when examining inflammatory markers and insulin signaling in traditional metabolically active tissue (e.g., liver, skeletal muscle, adipose tissue), it was observed that inflammation was evident in both skeletal muscle and liver in the high-fat diet fed group by week eight. In adipose tissue, inflammation within the high-fat diet group was not significantly visible until week 14. Similarly, when investigating cellular insulin resistance in liver, skeletal muscle, and adipose tissue within the high-fat diet fed group there were similar delays in noticeable dysfunction. By week eight, skeletal muscle and liver exhibited insulin resistance while insulin resistance was not established in adipose tissue until week 14. Taken together, over the course of a 14-week period investigating the effects of diet-induced obesity, both inflammation and cellular insulin resistance manifested in tissues essential for glucose homeostasis well after they became evident in the vasculature (12). The results from this study provided early evidence that the vasculature is more vulnerable to pernicious effects (i.e., insulin resistance, inflammation) of nutrient overload compared to other tissues and that the vasculature is a potential therapeutic target for combatting insulin resistance and increased CVD risk in the setting of T2D, a condition of chronic and sustained insulin resistance.

To translate previous findings in preclinical models that the development of vascular insulin resistance and inflammation precede the development of insulin resistance in metabolically active tissue, Smith *et al.* recently investigated the effects of transitioning from a healthy, active lifestyle to an obesogenic lifestyle (13). This included reductions in ambulatory activity and elevated nutrient consumption in young, healthy humans. Briefly, 36 (18 female) disease free, recreationally active, young, and normal weight individuals who regularly took >10,000 steps of walking per day and consumed less than 2 non-diet carbonated soft drinks per day were recruited to undergo an acute (10-day) obesogenic lifestyle. Throughout the study intervention, participants were asked to track and reduce their daily activity such that the aggregated steps walked per day was <5000, as assessed via a pedometer. Participants were also instructed to drink six cans of sugar-sweetened soft drinks (335 mL/can) daily and were otherwise allowed to eat *ad libitum*. Participants underwent pre (day 0) and post (day 10) test measurements to characterize their vascular phenotype, including measures of insulin-stimulated leg blood flow during a one-hour hyperinsulinemic-euglycemic infusion using physiological concentrations of insulin to mimic postprandial insulin concentrations, endothelial function as assessed via flow-mediated dilation (FMD) in the lower leg (popliteal) artery, and skeletal muscle microvascular perfusion. Additionally, measures of NO content and a lipid panel were assessed. Major findings from the study were that an acute adoption of an obesogenic lifestyle, defined as decreased ambulatory activity and increased caloric consumption, resulted in significant increases in bodyweight, body mass index, fasting insulin concentrations, and homeostatic model assessment for insulin resistance (HOMA-IR) values, all important contributors to the development of insulin resistance.

Interestingly, and for the first time documented in humans, a sexual dimorphism in the pathogenesis of vascular insulin resistance was observed (13). That is, there were significant reductions in insulin-stimulated leg blood flow and skeletal muscle microvascular perfusion in young healthy men, but not in women. Additionally, endothelial function, as assessed via popliteal FMD, had a trend, but not statistically significant, towards attenuation (P=0.06) in males, but not females. Furthermore, nitrite concentrations, a surrogate index of NO bioavailability, were significantly decreased in men, but not women. Lastly, when comparing blood glucose concentrations and glucose infusion rates during the one-hour hyperinsulinemic-euglycemic infusion, men, but not women, exhibited elevated blood glucose concentrations and impaired glucose infusion rates, providing the first piece of evidence that vascular insulin resistance in humans can be incited by acute adverse lifestyle changes in men.

Mechanisms of impairments in the vasculature of T2D

While insulin resistance (i.e., hyperglycemia) increases RAS/MAPK/ET-1 signaling pathways to induce endothelial dysfunction and impair both capillary recruitment and insulin-stimulated vasodilation, it is also associated with increased inflammation, oxidative stress, and atherosclerotic plaque development (8, 88). These various factors, while different, act via similar mechanisms to reduce the amount of NO produced and/or increase the amount of ET-1 released during insulin stimulation. Hyperglycemia, a classic feature of T2D, has been shown to directly induce endothelial dysfunction via oxidative stress and advanced glycation end products (AGEs) (106). Briefly, hyperglycemia increases the conversion of glucose into fructose via the polyol signaling pathway (107). Fructose reacts with proteins and lipids to increase the production of AGEs, which promotes oxidative stress. AGEs can promote a vicious cycle of generating more free radicals, thus contributing to increased oxidative stress, but they can also indirectly stimulate the release of pro-inflammatory signaling molecules, such as interleukin-1, interleukin-6, and tumor necrosis factor-alpha (106). These hyperglycemic-induced pro-inflammatory molecules can create an environment that favors the production and release of ET-1 due to decreased PI3K/Akt signaling, but also oxidative stress, causing low-grade chronic inflammation (8, 15), commonly found in those with T2D. This inflammatory milieu can lead to endothelial dysfunction with subsequent plaque development in the endothelium known as atherosclerosis.

With endothelial dysfunction, anomalies in the generation or bioavailability of NO promote a pro-inflammatory/pro-atherogenic phenotype, an early occurrence in the development of atherosclerosis. Under these conditions, endothelial cells secrete more pro-inflammatory molecules, increase vascular permeability, and increase expression of cell surface adhesion molecules, such as vascular cell adhesion molecule (VCAM-1) (108). With increased permeability, lipoproteins, such as low-density lipoproteins (LDL), enter into the circulation to become oxidized via oxidative stress (109). Once LDL molecules are oxidized, they are taken up by macrophages in the endothelium, forming foam cells. This foam cell accumulation signals inflammatory molecules and VCAM-1 to attract and bind immune cells (e.g., monocytes), contributing to the plaque development (109). Eventually, if left untreated, lesions of plaque development (i.e., atherosclerosis) form throughout the walls of arteries, which restricts blood flow due to a progressive narrowing of arterial lumen diameter, further contributing to impaired vascular insulin signaling. To experimentally demonstrate this concept, Rask-Madsen *et al.* studied apolipoprotein E null mice, a mouse model of atherosclerosis, to determine the *in vivo* significance of insulin signaling by genetically deleting, or leaving intact, insulin receptors located within endothelial cells (31). Insulin sensitivity, glucose tolerance, plasma lipids, and blood pressure were assessed in both groups with no significant differences observed. However, the authors demonstrated a roughly twofold increase in atherosclerotic lesions in the aorta, reduced endothelium-dependent vasodilation, increased expression of VCAM-1, and increased monocyte binding to endothelial cells. When VCAM-1 was blocked, monocyte adhesion to endothelial cells was reduced to below control values. Cumulatively, these results provided evidence that loss of protective insulin signaling in endothelial cells, in the absence of other competing systemic risk factors, accelerates atherosclerotic plaque development (31).

Conversely, maintenance or upregulation of the IRS/PI3K/NO signaling cascade protects against atherosclerotic plaque development (110). Kanter *et al.* investigated in LDL receptor deficient mice, a mouse model of metabolic syndrome, the effects of normal or diabetogenic chow (0.15% cholesterol) in three different, randomized groups receiving injections twice-daily of vehicle, insulin, or S597, a drug to selectively activate mostly the IRS/PI3K/NO portion of the insulin signaling cascade (110). It was noted that the S597-treated mice developed fewer atherosclerotic plaque lesions compared to mice treated with either insulin or vehicle injections. This is likely, in part, due to the selective activation of the protective, antiatherogenic IRS/PI3K/NO signaling cascade and the lack of inflammatory, proatherogenic RAS/MAPK/ET-1 signaling. Additionally, the observed reductions in atherosclerotic plaque lesions in mice treated with S567 is also attributed to the sluggish accumulation of immune cells responsible for plaque lesion development and progression.

Overall, a balance between protective IRS/PI3K/NO and inflammatory RAS/MAPK/ET-1 insulin signaling in the vasculature is essential for maintenance of endothelial function and prevention of CVD risk factors. In patients with insulin resistance or T2D, selective insulin resistance and endothelial dysfunction are implicated in the pathogenesis of cardiovascular and metabolic disease. Therapeutic strategies to ameliorate vasculo-metabolic derangements are paramount in improving health outcomes in this growing population.

Lifestyle strategies to improve vasculo-metabolic health

Healthy lifestyle choices and diet are the cornerstone for reducing the risk of developing obesity, T2D, and CVD (111). It has been demonstrated that dietary choices have significant effects on vascular function and metabolic health (12, 19, 111–114). Indeed, epidemiological evidence confirms that diets rich in fruits and vegetables promote metabolic health, improve endothelial function, and prevent the development of CVD (115–117). For example, countries who border the Mediterranean Sea (e.g., Greece, Italy, and Spain) typically consume a mediterranean diet, that is the consumption of lean fish, raw vegetables, and fewer high-fat food options, which results in having a lower prevalence of CVD (118) compared to countries that consume a Western diet, which is nutrient-poor and rich in calories, saturated fat, sodium, and fructose (119). According to the American Heart Association, diets consisting of greater than 35% of total calories from fat is classified as a high-fat diet. There is no doubt that high-fat and high-sugar diets are associated with an increased risk of CVD, as previously elucidated (12). Additional diet-related studies have documented further evidence of metabolic and lipid profile abnormalities (120) and direct effects of endothelial dysfunction induced by high dietary fat and sugar intake (121). This type of diet composition, commonly manifested within a Western diet, is considered a risk factor for CVD due to its' strong association with obesity and insulin resistance (122).

Previous work has shown that, in humans, insulin resistance in obesity and T2D contributes to impaired glucose disposal and to the development of vascular dysfunction and atherosclerotic disease (31, 97, 110). To this end, prior evidence suggests that a six-month low-glycemic, calorie-restricted (500 kcals) dietary intervention designed to induce weight loss by reducing intake of dietary sugars <5% of energy was sufficient to significantly reduce bodyweight, HOMA-IR, chronic inflammation, and significantly improve metabolic outcomes and vasodilatory responses to insulin stimulation (19). One of the mechanisms that potentially explain the improvements in vasculo-metabolic health observed with dietary interventions is caloric restriction. Calorie restriction is one of the most potent dietary interventions that not only is reproducible, but also shows beneficial effects in extending lifespan and reducing the risk of developing obesity and endothelial dysfunction (123). Briefly, caloric restriction, in addition to improving traditional cardiovascular risk-factor profiles, has shown to reduce oxidative stress (i.e., inflammation), inflammatory cytokines, and upregulate sirtuin 1 expression, which can stimulate eNOS expression and activity (124, 125). To substantiate, inhibition of sirtuin 1 prevents endothelium-dependent vasodilation and reduces NO bioavailability, suggesting that NO-mediated effect of caloric restriction is regulated, in part, by both sirtuin 1 and decreases in pro-inflammatory molecules (126). Other beneficial effects of caloric restriction that have been noted are improvements in insulin sensitivity and reduced incidence of diabetes diagnosis in obese adults (127) and reduced blood pressure in adults who regularly consume a Western diet (128). Taken together, the importance of dietary choices in combating vasculo-metabolic derangements are of paramount importance when prescribing lifestyle modifications, especially for individuals present with obesity and insulin resistance.

Another key component for a healthy lifestyle is participation in regular exercise. While overconsumption of calorie-rich foods have been identified as a risk factor for the development of various cardiovascular complications (129, 130), a sedentary lifestyle is also a key risk factor gaining more public attention (131). Regular aerobic exercise for adults has long been defined as 150 to 300 minutes of moderate intensity or 75 minutes of vigorous intensity weekly with moderate-to high-intensity resistance training at least two days per week, however, approximately 80% of adults are insufficiently active in the United States (45). Previous work indicates that even 10-days of inactivity results in decreased whole-body insulin sensitivity and can have profound declines in vascular insulin sensitivity (13).

On the contrary, numerous studies from animals and humans have documented that exercise is a potent stimulus for enhancing insulin-stimulated vasodilation and blood flow to skeletal muscle (29, 39–44). To this regard, Dela *et al.* reported that when healthy and T2D patients undergo a 10-week single-leg aerobic exercise intervention, the trained leg had significant improvements in insulin-stimulated conduit artery blood flow and skeletal muscle glucose uptake when compared to the non-trained leg (42). Interestingly, it was determined that these improvements were not elicited by a single bout of exercise, but rather, a physiological adaptation to repeated exercise. However, the effects are short-lived in patients with T2D, with beneficial changes gone within one week. This is in line with work from others showing that with cessation of exercise, adaptations to regular exercise are diminished quickly (13, 46, 132–137), suggesting the importance of consistent exercise sessions.

Resistance exercise has also shown improvements in vascular function and metabolic outcomes. Critical work performed by Holten *et al.* investigated whether a low weekly training volume (30 min, 3x/week) single-legged exercise intervention for six weeks could improve insulin action in skeletal muscle (47). Healthy versus T2D patients performed a one-legged machine-based training program that consisted of leg press, knee extension, and hamstring curl for three to four sets, 10-20 reps with intensity performed between 50-80% one-repetition max with the intensity increasing as weeks progressed. Results from the six-week intervention documented significant robust improvements in insulin-stimulated leg blood flow during a hyperinsulinemic-euglycemic clamp in both healthy and T2D patients within the trained leg. Furthermore, leg glucose clearance, protein content of GLUT4, insulin receptor, and protein kinase β, otherwise known as Akt, part of the vascular protective insulin signaling cascade, also significantly increased following the training intervention (47). These physiological adaptations from chronic exercise are essential for improvements or restoration of vascular function and metabolic outcomes in humans and in conjunction with other studies showing skeletal muscle insulin sensitivity improvements due to changes in muscle size, morphology, protein composition, and capillarization (58, 138).

Short-term exercise interventions have also shown improvements in the responsiveness of the vascular endothelium and glucose uptake. In fact, a single bout of exercise results in elevated insulin sensitivity and glucose uptake immediately following exercise and for up to 48 hours later (46). Mikus *et al.* corroborated endothelial responsiveness following an acute exercise intervention in overweight and obese T2D patients (41). Participants performed seven days of supervised treadmill and cycling exercise for 60min/day at 60-75% heart rate reserve with oral glucose tolerance tests performed pre- and post-exercise intervention. Prior to exercise, leg blood flow remained unchanged during the glucose ingestion, however, following the seven-day exercise intervention, postprandial leg blood flow during the oral glucose tolerance test in patients with T2D significantly improved, with rises in circulating insulin in response to glucose ingestion, without accompanying changes in metabolic outcomes (41).

Exercise has also been shown to improve the skeletal muscle microvasculature in rats with insulin resistance and T2D, without accompanying changes in metabolic outcomes. Further emphasizing the role of exercise as a therapeutic target to restore endothelial responsiveness, a rat model of obesity and T2D was investigated to test if chronic voluntary wheel running (RUN) prevented attenuations in insulin-stimulated vasodilation associated with obesity and T2D independent of RUN or adiposity (49). Briefly, Otsuka Long Evans Tokushima Fatty rats, a model of obesity and T2D, were randomized to either RUN, caloric restriction (CR; diet adjusted to match the bodyweight of RUN group), or sedentary control. At 40 weeks, isolated gastrocnemius second-order arterioles from RUN, CR, and sedentary groups were collected to assess insulin-mediated vasoreactivity via NO and ET-1 pathways. Tezosentan, an ET-1 receptor antagonist, was used to isolate NO's contribution, while [NG-nitro-l-arginine methyl ester (L-NAME)], a NOS inhibitor, assessed overall NO involvement. Metabolic markers (weight, fasting glucose, hemoglobin A1c) were significantly lower in RUN and CR compared to sedentary, but only RUN exhibited improved glucose tolerance area under the curve. Insulin-induced vasodilation was significantly enhanced in RUN versus other groups upon tezosentan administration, signifying a more prominent role for NO in this group. Notably, no significant differences in vasodilation were observed between groups with L-NAME coadministration, suggesting potential involvement of additional mechanisms beyond NO in insulin-mediated vasodilation.

Based on these findings, it was concluded that daily exercise prevented obesity and T2D-associated impairments in insulin vasodilatory responses within skeletal muscle arterioles via NO-mediated mechanisms that were independent of adiposity (49). These results have been translated into healthy humans that underwent a single session of one-legged exercise with a hyperinsulinemic-euglycemic clamp assessed four hours after cessation of exercise (39). Following a 60-minute exercise bout, microvascular perfusion was assessed via ultrasonography in the vastus lateralis of each leg with a 65% improvement noted in the exercised leg versus 25% in the rested, control leg. Additionally, skeletal muscle glucose uptake was enhanced in the exercised leg compared to the rested leg, however, when a NO synthase inhibitor was infused, the augmented effect of exercise-mediated improvements in insulin-stimulated microvascular blood flow and glucose uptake were blunted. These findings demonstrate the parallel improvements between the vasculature and skeletal muscle are NO-dependent (39).

To summarize, studies from humans and animals indicate that exercise can improve the vascular endothelium's responsiveness to insulin not only at the level of the conduit artery, but also in the skeletal muscle microvasculature. This suggests that exercise triggers a mechanism, independent of metabolic changes, to prime the vasculature's ability to dilate in response to insulin. Further research is needed to identify the specific mechanisms by which exercise exerts these beneficial effects on the vascular endothelium to offer potential therapeutic avenues in individuals present with endothelial dysfunction, commonly found in those with insulin resistance and obesity, among other disease states.

The mechanistic role of shear stress as a vascular-sensitizing agent

Numerous studies from animals and humans illustrate the potency of exercise to augment insulin-stimulated vasodilation and skeletal muscle blood flow (29, 39–44). A consistent and critical finding is that the insulin-sensitizing effect on the vasculature appears to be restricted to vascular beds that experience increases in blood flow during exercise, independent of changes in body composition or reductions in hyperglycemia (39–41). It is suggested that the underlying mechanism to exercise-induced increases in the vascular endothelium's responsiveness to insulin is due to increases in shear stress, or the tangential force of blood flow on the endothelial surface in blood vessels (51).

Direct evidence from cell culture shows when endothelial cells are subjected to shear stress for three hours there are significant increases in eNOS expression that remain elevated for 12 hours post-flow exposure (52). This finding was recapitulated in isolated perfused arteries from rats exposed to high flow versus no flow (53). Arteries taken from rat soleus muscles were exposed to four hours of high flow versus no flow conditions with the conjecture that elevated intraluminal shear stress upregulates eNOS expression and improves endothelium-dependent vasodilation via increased NO production. Following the four-hour treatment period, eNOS mRNA expression was investigated and determined to be augmented in arteries treated with high flow compared to no flow conditions (53). Subsequently, in a separate set of experiments, arteries pretreated with high flow conditions underwent endothelium-dependent vasodilatory assessments via addition of progressive acetylcholine doses in the bath solution (53). Results from this experiment showed that arteries pretreated with high flow (i.e., shear stress) intraluminally improved ACh-induced dilation, thus corroborating the findings that shear stress augments eNOS expression and enhances endothelium-dependent vasodilation (53).

Importantly, findings from cell culture and isolated vessels from rats were translated in humans undergoing local arm heating without exercise (54), only lower-body exercise (55), and only upper-body handgrip exercise (56). In these unique studies, a novel, non-invasive approach to examine shear stress responses and vascular function in both arms before and after the intervention was implemented. During the intervention phase of these studies, one arm was occluded with a blood pressure cuff, preventing blood flow and shear stress increases above resting values, whereas the other arm was not occluded, allowing the arm to fully experience increases in blood flow and shear stress stimuli. Arterial responses in both the conduit artery and microvasculature were assessed via brachial artery FMD and cutaneous microvascular endothelial function, respectively. Results from these studies indicated that the occluded arm did not significantly dilate or adapt to the intervention whereas significant vasodilatory responses were observed in the non-occluded arm demonstrating that changes in shear stress, under rest and during exercise, provide the principal physiological stimulus for vascular remodeling and endothelial responsiveness (54–56).

Moreover, cell culture studies show that shear stress is capable of reducing the expression of ET-1 in a dose-dependent fashion (139). In this study, cultured endothelial cells were subjected to shear stress of different intensities for 24 hours. It was documented that ET-1 concentrations were downregulated following 24 hours of shear stress application, however, when eNOS was inhibited, the reductions in ET-1 concentrations were prevented. This data suggests that the shear stress-induced downregulation of ET-1 in cultured endothelial cells is NO-mediated. This is in line with the proposed IRS/PI3K/eNOS and RAS/MAPK/ET-1 signaling pathway, whereas the amount of signaling present on one side influences the signaling content on the other side (29). In other words, reductions in ET-1 can suppress inflammatory/proatherogenic environments and with less ET-1, there are increases in NO bioavailability, promoting a vascular protective phenotype, which may be one of the primary mechanisms in which exercise renders the vascular endothelium more sensitive. Not only does shear stress prime the endothelium to be more responsive, but it also amplifies insulin signaling and subsequent insulin-stimulated vasodilation with (29, 39, 49) and without (50) exercise. Previous work shows when cultured endothelial cells, isolated arteries, and humans undergo one hour of shear stress stimulation, insulin signaling with subsequent increases in insulin-stimulated vasodilation are enhanced (50). This increase in insulin-stimulated vasodilation is typically associated with significant increases in microvascular perfusion (50, 57) and skeletal muscle glucose uptake (46, 58, 59), depending on the severity of dysfunction.

Taken together, previous evidence suggests that exercise increases endothelial shear stress-induced vascular adaptations and that the vascular actions of insulin and shear stress appear to be mediated via increases in NO bioavailability. Indeed, shear stress enhances eNOS content and reduces the production of ET-1 in cell culture and isolated arteries. Additionally, shear stress and insulin activate eNOS through the IRS/PI3K signaling pathway (29). These effects are one of the proposed mechanisms in which a therapeutic modality targeting the vascular endothelium, such as exercise, can improve insulin sensitivity and reduce overall CVD risk.

The vascular endothelial glycocalyx as a therapeutic target for combatting CVD

Current standards of medical care for individuals with T2D is emphasizing lifestyle modifications, such as increased exercise participation, as a first-line therapy to manage T2D and decrease CVD risk (20). Results from a large clinical trial of 5000+ T2D participants across ~10 years revealed that intense weight loss focused lifestyle interventions did not reduce CVD morbidity or mortality (20). This discovery, along with other pieces of evidence from independent labs (21–24), show precedence that exercise training in patients with T2D does not evoke optimal exercise adaptations in the vasculature. Further, a recent meta-analysis revealed that exercise-induced improvements in endothelial function in patients with T2D are blunted compared with non-diabetics (21). Interestingly, blunted vascular adaptations following exercise in patients with T2D may also be a contributing mechanism to the diminished cardiorespiratory fitness gains reported (25). These factors may explain why exercise does not substantially reduce CVD morbidity and mortality in patients with T2D, warranting a better understanding of the mechanisms responsible for such restrictions and identifying new adjuvant therapies targeting the dysfunction observed in the vasculature.

Starting from within and proceeding outwardly of all arteries are three distinct layers, the tunica intima, tunica media, and tunica adventitia. The blood vessel's innermost layer, the tunica intima, houses a specialized layer of endothelial cells called the endothelium. It is here that the endothelial cells directly make contact and are oriented in the direction of the flowing blood. The middle layer, tunica media, is comprised of VSMC’s, which are responsible for regulating arterial diameter acutely via contraction or relaxation of smooth muscle cells. Lastly, the tunica adventitia, made of mostly collagen and elastic fibers, is for structural rigidity of the vessel and helps support growth and repair of the arterial wall. Recently, heavy emphasis has been placed on the vascular endothelial glycocalyx, a functional structure consisting of negatively charged proteoglycans, glycoproteins, and glycosaminoglycans found in the luminal side of the vascular endothelial cells, for improving vascular outcomes in T2D (61, 62). These fine, hair-like structures located throughout the arterial tree detect blood flow-induced shear stress and convert shear forces into signals to synthesize components of itself while also producing signaling molecules that provide messages to the underlying VSMC's, including vasoconstriction, vasodilation, remodeling, and inflammation (61).

Previous reports have indicated that a thick glycocalyx represents a healthy, vascular protective phenotype while an impaired, or thin, glycocalyx represents a damaged phenotype (Figure 2.3), predisposing the vascular endothelium to endothelial dysfunction and cardiovascular disease (60–64). Additionally, evidence suggests that the glycocalyx is imperative in mechano-transduction of shear stress-induced NO release for the full effects of post-exercise vascular adaptations to be observed. However, in T2D, the glycocalyx is impaired and heavily degraded, limiting the potential for exercise-induced vascular adaptations (60). Efforts to study, repair, and resynthesize the glycocalyx show promise to rectify endothelial dysfunction and subsequent insulin-stimulated vasodilation in patients with T2D. Key components of the glycocalyx include, but are not limited to, hyaluronic acid receptor (CD44), syndecan-1, glypican-1, heparan sulfate, hyaluronan (or hyaluronic acid), and VCAM-1 (61). CD44 attaches indirectly to endothelial cells, providing structural support to the glycocalyx. Syndecan-1 and glypican-1 are proteoglycans that connect to the cell membrane and play a part in transmission of extracellular mechanical forces and mediates shear stress mechano-transduction signaling pathways, such as eNOS synthesis and NO production (140, 141). Heparan sulfate is the dominant glycosaminoglycan found on the surface of the endothelium, comprising 50-90% of all glycosaminoglycans attached to proteoglycans (61) and makes up the physical and functional characteristics of the glycocalyx, including aiding in shear stress-induced eNOS activation (141). Another proteoglycan, hyaluronan, attaches to endothelial cells via CD44 and triggers both NO producing and inflammatory pathways by balancing space between flowing blood and vessel wall (i.e., glycocalyx), mechanotransduction, and by acting as a selective permeability barrier due to its negative charge, preventing adhesion molecules such as VCAM-1 from sticking to vessel walls (61, 142).

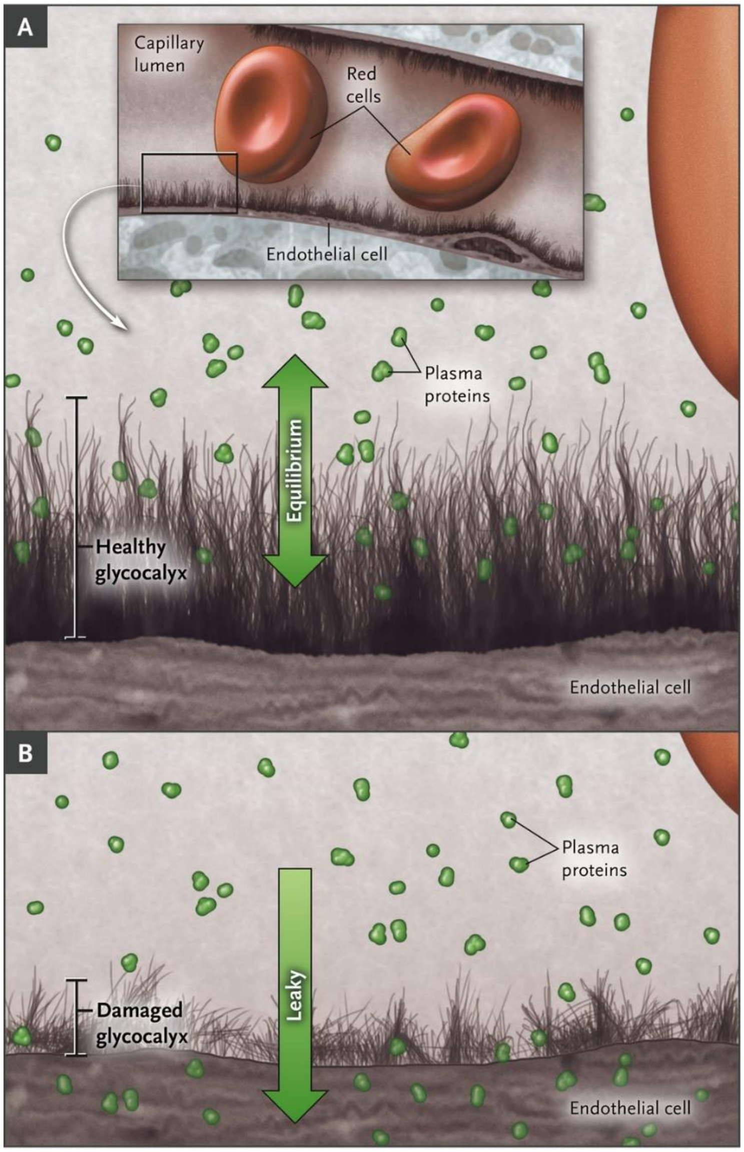

Figure 2.3. Endothelial glycocalyx layer in health (A) and in disease (B). The endothelial glycocalyx is located on the apical surface of the vascular endothelium. The endothelial glycocalyx acts as a dynamic sensor, constantly bombarded by variations in blood flow and plasma proteins. These blood flow variations can deform, stimulate, or shed glycocalyx components resulting in diverse outcomes, such as activation of eNOS with subsequent induction of

vasodilation or decreased NO bioavailability with subsequent vasoconstriction. Adapted from *Myburgh GA, Mythen MG*. (143).

The endothelium endures persistent distortions from the blood flow's pressure and shear stress. Prior research has indicated that removal of key glycocalyx components, or the entire structure, demonstrates its critical function as a mechanosensor and translating mechanical forces exerted by blood flow into signaling cascades. Indeed, in glycocalyx-denuded cells and blood vessels, shear-induced NO production is lacking (66). Several key players within the glycocalyx contribute to its ability for mechanosensation and mechanotransduction of shear stress including heparan sulfate, glypican-1, hyaluronan, and sialic acid (66, 144–147), a nine-carbon sugar molecule that attaches to glycoproteins (61), however, their individual contribution may depend on the specific endothelial cell type or vascular bed studied. As a matter of fact, the overall structure (e.g., its length) of the glycocalyx also plays a role in mechanotransduction. Decreased glycocalyx length has been positively correlated with blunted endothelial function, including eNOS synthesis and NO production (65). Evidence shows that in animal models and humans with T2D that the endothelial glycocalyx is degraded both structurally and functionally (65, 142, 148–152). In other words, the length of the glycocalyx in a T2D mouse model is reduced (65) and this is accompanied by a reduction in the sensing ability of the glycocalyx to transduce shear forces into vasodilatory responses (65, 148–150, 152).

Another function of the negatively charged glycocalyx is to provide an electrostatic barrier between negatively charged molecules and cells, including erythrocytes and activated platelets, from making contact with the vessel wall (Figure 2.4). This barrier acts as one of the first lines of defense against negatively charged pathogens floating in the bloodstream from making contact with the endothelium and infecting the underlying tissues. However, under pathological conditions, such as T2D, hypertension, advanced age, inflammation, and hypercholesterolemia, the electrostatic barrier of the glycocalyx shrinks due to the loss of glycocalyx integrity (i.e., reduced length) (61). This allows deeper erythrocyte penetration, which is associated with decreased capillary perfusion (64).

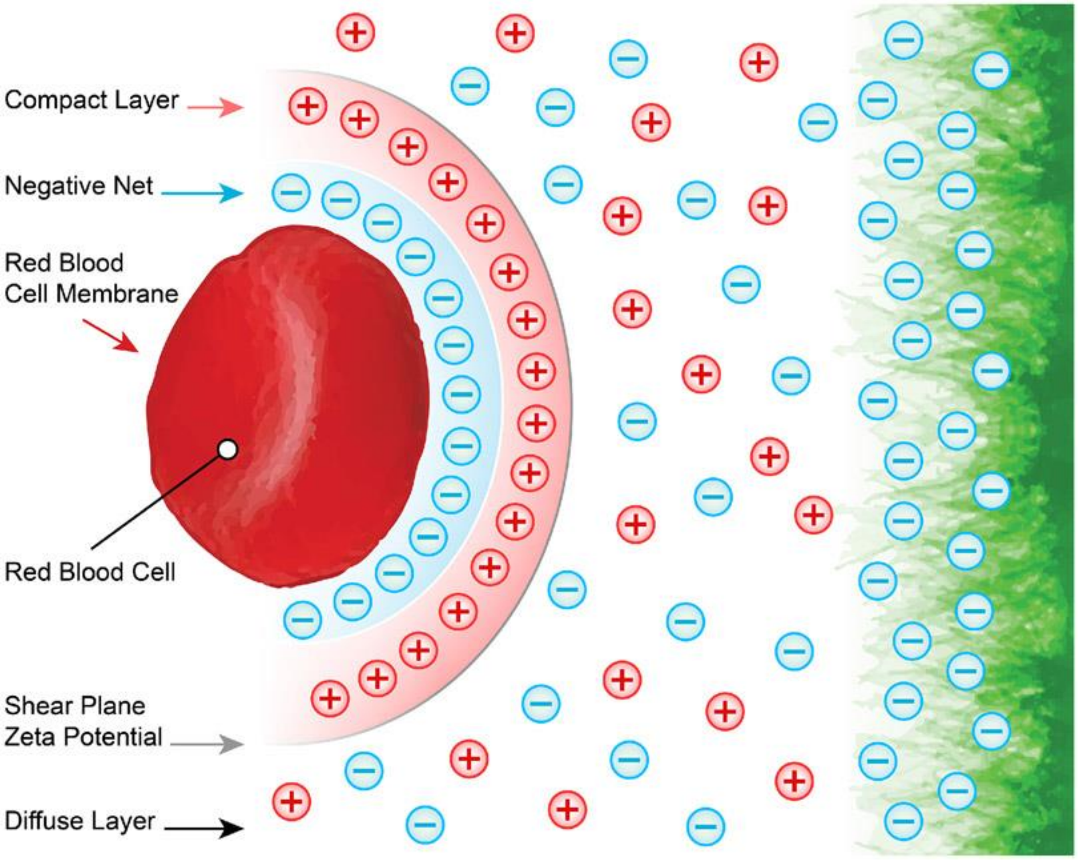

Figure 2.4. The Endothelial glycocalyx is negatively charged and functions as a repellent between the endothelium and negatively charged molecules, such as erythrocytes. Adapted from Foote *et al.* (61).

Under nonpathological conditions, erythrocytes flow in a column pattern in the vasculature and do not make contact with the endothelium, as they are separated by the protruding glycocalyx. The distance between the erythrocytes and their small penetration in the glycocalyx is called the perfused boundary region (PBR) (153, 154), as shown in Figure 2.5. An increased PBR represents deeper penetration of erythrocytes into the glycocalyx, denoting its degradation and thinning (155). This PBR value can be assessed *in vivo* non-invasively in humans using sidestream darkfield imaging (64) by placing a camera sublingually to examine the microvasculature. Prior evidence shows that flowing erythrocytes maintain a fixed distance from the endothelium in health, however, under pathological conditions when the glycocalyx becomes damaged/shorter, the erythrocytes penetrate deeper into the glycocalyx, thus increasing the PBR (64).

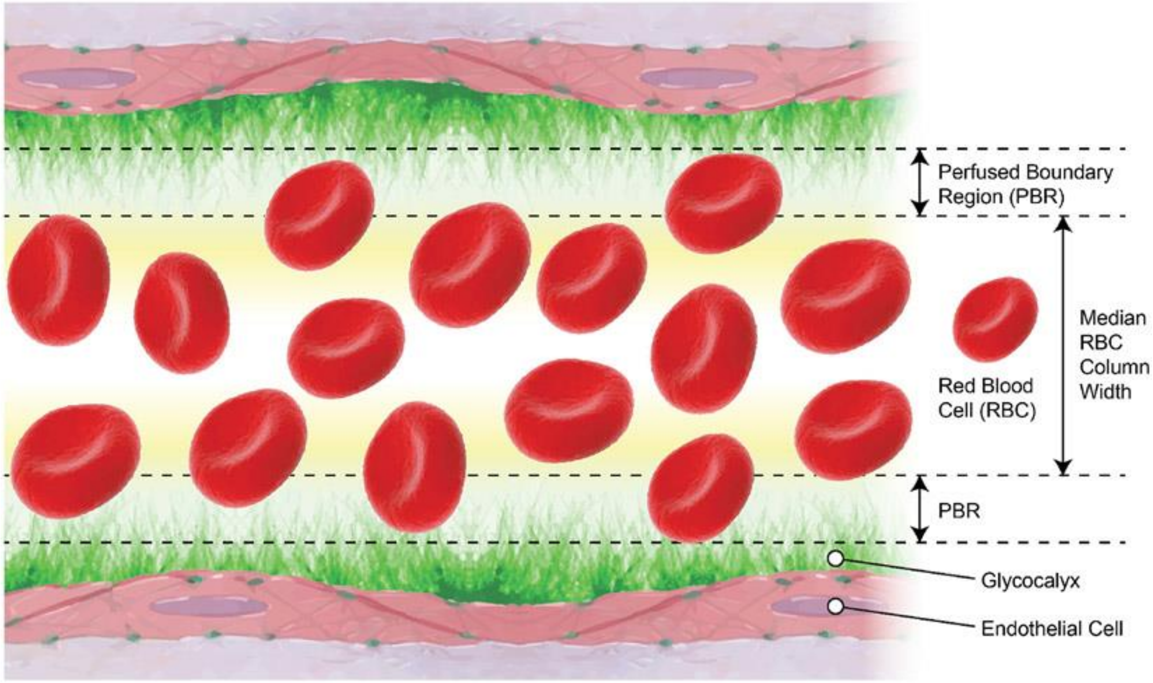

Figure 2.5. In health, erythrocytes maintain a fixed distance as they flow throughout the vasculature with little penetration into the glycocalyx. Under pathological conditions, the erythrocytes penetrate deeper and flow closer to the endothelium due to shortened glycocalyx length, increasing the PBR. Adapted from Foote *et al.* (61).

Type 2 diabetes is characterized by chronic hyperglycemia, which has shown deleterious effects on the glycocalyx due to oxidative stress and AGE's (150, 156). Additionally, insulin resistance and the associated inflammatory state further exacerbate glycocalyx integrity degradation (156). Previous evidence shows that in T2D the endothelial glycocalyx length is reduced and PBR is increased (152). Therapeutic interventions aimed at restoring the length of the glycocalyx, with subsequent decreases in PBR are underway in various clinical trials. The market currently offers two nutraceuticals, or dietary supplements, Arterosil® and Endocalyx®, that have been designed to restore the integrity of the endothelial glycocalyx (67, 157). Arterosil® was the first nutraceutical developed with the intention of protecting and restoring the endothelial glycocalyx. This compound contains the polysaccharide rhamnan sulfate, a heparin and heparan sulfate mimetic extracted from the *Monostroma nitidum* algae (67). Arterosil® was first demonstrated efficacious within a cell culture study where human coronary artery endothelial cells were treated with rhamnan sulfate and exhibited significant increases in heparan sulfate coverage compared to untreated cells (158). The treated cells also exhibited an improved electrostatic barrier resulting in decrease permeability to negatively charged molecules such as LDL (158). In humans, Arterosil® has shown to repair glycocalyx damage from the deleterious impact of hyperglycemia by limiting adhesion molecules to the endothelium, improving endothelial-dependent elasticity, and reducing the number of atherosclerotic plaque lesions (67). While Arterosil® has shown beneficial results thus far, the literature in human clinical trials is limited, and hesitancy is expressed due to the ingredient rhamnan sulfate. This ingredient is not naturally found in the body and may result in an overactive immune response for some individuals. Nevertheless, based on the history of Arterosil®, it shows promise and future studies are warranted in human populations.

In 2018, Endocalyx® joined Arterosil® as a second nutraceutical option for protecting and restoring the endothelial glycocalyx (67). Endocalyx® contains hyaluronic acid, glucosamine for heparan sulfate and hyaluronic acid synthesis, antioxidants, and fucoidan, a heparan sulfate-like component (67). With these different components, Endocalyx® is a multistrategy therapeutic with high expectations for successful outcomes. Previous evidence in mice have shown that 10-weeks of dietary Endocalyx® supplementation decreased PBR and improved the electrostatic barrier in aged-mice compared to young, healthy, control mice (68). Additionally, NO bioavailability was augmented in carotid arteries of treated mice with improvements in endothelium-dependent vasodilation and reductions in aortic stiffness compared to controls. These data suggest that Endocalyx® regenerates and restores the endothelial glycocalyx.

Endocalyx® supplementation to restore the endothelial glycocalyx in humans is limited. In older adults, three months of Endocalyx® supplementation did not restore overall FMD or glycocalyx length compared to placebo (69). Additionally, in individuals not taking antihypertensive medications, Endocalyx® improved capillary glycocalyx thickness more so than placebo. These data suggest that in older adults, Endocalyx® may have positive outcomes on capillary glycocalyx thickness, but further investigation is warranted. In South-Asian Surinamese descent individuals with T2D (n=19), three months of

Endocalyx® supplementation improved inflammatory markers and microvascular endothelial health compared to a diet intervention, however, future randomized clinical trials with larger sample sizes are warranted (70). These discrepancies in results may be due to ethnic differences or the impact of potential different usage rates of antidiabetic agents, such as sodium-glucose cotransporter-2 (SGLT2) inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists, with known cardiovascular benefits (159, 160). It is unsure if Endocalyx® would improve other indices of vascular health in T2D, such as capillary perfusion, arterial stiffness, and endothelium-dependent vasodilation *in vivo* at this time.

In summary, T2D rates are increasing by drastic proportions worldwide. T2D is characterized by insulin resistance, impaired vascular function, and decreased insulin signaling in the vasculature. Lifestyle interventions, such as exercise, have proven ineffective in combatting vascular derangements found in T2D. Due to these findings, novel adjuvant therapies are of paramount importance. Based on the preclinical success of Endocalyx® in restoring the integrity of the glycocalyx, this area of research shows extraordinary promise for improving vascular health *in vivo* in humans. Collectively, DSGP (i.e., Endocalyx®) shows potential, but more studies are warranted. Based on the gaps in the literature, this dissertation will determine if endothelial glycocalyx integrity, insulin-stimulated vasodilation, and endothelium-dependent vasodilation are improved following DSGP in individuals with T2D. Furthermore, this dissertation will determine the efficacy of Endocalyx® as a viable therapeutic agent in combatting vasculo-metabolic derangements found in the setting of insulin resistance and obesity.

CHAPTER 3: MAIN FINDINGS

Impact of dietary supplementation of glycocalyx precursors on vascular function in type 2 diabetes

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Running title: Glycocalyx and Vascular Function in Type 2 Diabetes

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ABSTRACT

Degradation of the endothelial glycocalyx in type 2 diabetes (T2D) is thought to contribute to impaired shear stress mechanotransduction, leading to endothelial dysfunction and the development of cardiovascular disease. Herein, we tested the hypothesis that restoration of the endothelial glycocalyx with dietary supplementation of glycocalyx precursors (DSGP, containing glucosamine sulfate, fucoidan, superoxide dismutase, and high molecular weight hyaluronan) improves endothelial function and other indices of vascular function in T2D. First, in db/db mice, we showed that treatment with DSGP (100 mg/kg/day) for four weeks restored endothelial glycocalyx length, as assessed via atomic force microscopy in aortic explants. Restoration of the glycocalyx with DSGP was accompanied by improved flow-mediated dilation (FMD) and reduced arterial stiffness in isolated mesenteric arteries. Further corroborating these findings, treatment of cultured endothelial cells with that same mixture of glycocalyx precursors promoted glycocalyx growth. Next, as an initial step to investigate the translatability of these findings, we conducted a pilot (n=22) double-blinded randomized placebo-controlled clinical trial to assess the effects of DSGP (3,712.5 mg/day) for eight weeks on endothelial glycocalyx integrity and indices of vascular function, including FMD, in Veterans with T2D. Contrary to the hypothesis, DSGP neither enhanced endothelial glycocalyx integrity nor improved vascular function indices relative to placebo. Together, these findings conceptually support the notion that restoration of the endothelial glycocalyx can lead to improvements in vascular function in a mouse model of T2D; however,

DSGP as a therapeutic strategy to enhance vascular function in individuals with T2D does not appear to be efficacious.

INTRODUCTION

The prevalence of type 2 diabetes (T2D) continues to increase in the United States and worldwide (2, 161, 162). In particular, T2D is widespread among the Veteran population, and the Veterans Affairs (VA) administration spends $1.5 billion annually on diabetes care (3, 4). Importantly, T2D contributes to the staggering rates of cardiovascular disease (CVD) and cardiovascular mortality in this population (5). Indeed, it is estimated that eight out of 10 patients with T2D will die from CVD (163). Endothelial dysfunction, a central vascular feature of T2D, plays a vital role in the pathogenesis of CVD (164–167). Thus, a better understanding of the mechanisms underlying endothelial dysfunction in T2D can be exploited for therapeutic gain in preventing and treating CVD.

The endothelial glycocalyx, a negatively charged layer of membrane-bound glycoproteins, proteoglycans, and glycosaminoglycans located on the apical surface of vascular endothelial cells, plays an essential role in transmitting mechanical forces (*i.e.*, shear stress) into the cell (61, 66). This process, known as mechanotransduction, is critical for maintaining endothelial health, such that if impaired, it leads to dysfunction of the endothelium. Indeed, degradation of these luminal mechanosensing structures, which occurs in T2D owing to oxidative stress, inflammation, hyperglycemia, disturbed or reduced flow profiles, among other mechanisms (61, 142, 150, 151, 168–170), contributes to impaired shear stress mechanotransduction and, consequently, endothelial dysfunction (60, 65, 142). Therefore, targeting of endothelial glycocalyx degradation can be considered a promising therapeutic strategy for combating vascular complications in T2D.

To that end, we report that restoration of the endothelial glycocalyx with dietary supplementation of glycocalyx precursors (DSGP, containing glucosamine sulfate, fucoidan, superoxide dismutase, and high molecular weight hyaluronan) increases glycocalyx length, improves flow-mediated dilation (FMD), indicative of enhanced shear stress mechanotransduction, and lessens arterial stiffness in a mouse model of T2D (*i.e.*, db/db mice). Further supporting these findings, we show that treating cultured endothelial cells with this cocktail of glycocalyx precursors promotes glycocalyx formation. Based on these observations, we proceeded to conduct a double-blinded, randomized, placebo-controlled clinical trial to examine the effects of eight weeks of DSGP on endothelial glycocalyx integrity and indices of vascular function, including FMD, in Veterans with T2D. We hypothesized that, compared to placebo treatment, DSGP would enhance the integrity of the endothelial glycocalyx and improve overall vascular function in Veterans with T2D.

METHODS

Animal Studies

All animal procedures were approved by the Animal Use and Care Committee at the University of Missouri and performed in accordance with

National Institutes of Health guidelines. 12-week-old db/db female mice were obtained from The Jackson Laboratory. Mice were allocated to two treatment groups: Dietary supplementation of glycocalyx precursors (DSGP; Endocalyx®) 100 mg/kg/day or vehicle (*i.e.*, peanut butter) for four weeks. Age-matched db/+ cohort was used as a reference control. Standard chow for feeding (5053-PicoLab Rodent Diet 20, LabDiet) was provided and mice had unlimited access to water. Two mice were housed in each cage under 12-hour light and dark cycles. Caretakers monitored the health of the mice daily. For euthanization, isoflurane inhalation (2%, AKORN Animal Health) with room air (250 mL/min) was used to achieve surgical anesthesia, followed by pneumothorax and exsanguination. Arteries were removed for functional and mechanical assessments. Each mouse was considered an experimental unit for analyzing all study outcomes.

Ex vivo Aortic Vasomotor Function

Aortic 2-mm rings were prepared by removing perivascular fat and connective tissue and then were mounted in 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 kept at 37°C, as previously described (171). To assess viability, arterial rings were constricted with KCl (80 mM). Aortas were pre-constricted with the prostaglandin H2/thromboxane A2 receptor agonist, U-46619 (20 nM), following washout. Cumulative additions of acetylcholine (ACh, 10^{-9} to 10^{-5} M) and the NO-donor sodium nitroprusside (SNP, 10^{-9} to 10^{-4} M) agonists were added to the vessel bath to determine arterial relaxation responses. ACh was used to assess endothelium-dependent relaxation while SNP was used to assess endothelium-independent relaxation.

Ex vivo Arterial Vasomotor and Mechanical Responses

Mesenteric arteries were collected for vascular functional and mechanical responses via pressure myography following euthanasia, as previously described (171, 172). The isolated arteries were cannulated and mounted onto pressure myographs set at an intraluminal pressure of 70 mmHg and exposed to a series of sequential 10^{-5} M phenylephrine pre-constrictions followed by increasing concentrations of ACh (10^{-9} to 10^{-5} M) or SNP (10^{-8} to 10^{-4} M) in half-log increments every two minutes to assess endothelium-dependent and -independent vasodilatory responses. Following this, the cannulated vessels were exposed to a calcium-free buffer containing 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and 0.1 mM adenosine to achieve passive conditions. Intraluminal pressures were then varied from 5 to 120 mmHg at intervals of two minutes, with internal diameter and wall thickness recorded to assess vascular remodeling and stiffness, as described previously (171, 172). For detailed equation descriptions and parameters implemented to assess mechanical and biophysical properties of arteries, see Wenceslau *et al.,* 2021 (173). Each artery originated from one mouse for outcome assessments.

Ex Vivo Endothelial Stiffness

The elastic modulus (stiffness) of the aortic endothelial surface was measured in explants of thoracic aorta from mice using atomic force microscopy (AFM, MFP-3D AFM 89 Asylum Research Inc., Goleta, CA), as described previously (171, 174). Briefly, a segment of the thoracic aorta is isolated and opened longitudinally and secured onto a glass slide with Cell-Tak adhesive. The samples were maintained at room temperature (~25°C) and subjected to repeated cycles of nanoindentation and retraction. A custom Python script analyzed the generated indentation-curves to calculate the force-curves and determine the elastic modulus of the endothelial surface using the Hertz model (152).

Ex Vivo Glycocalyx Integrity

Similar to the method above to measure the elastic modulus, endothelial glycocalyx length and stiffness in aortic explants were assessed via AFM nanoindentation experiments using a 5.0 μm spherical probe attached to a cantilever (NovaScan, USA), with a spring constant of 0.02 N/m. To perform the curve analysis needed to determine glycocalyx length and stiffness, a custom code was implemented following the recommendations outlined by Targosz-Korecka *et al.*, 2017 (65). For each 2 mm^2 aortic explant, at least 50 nanoindentation curves were obtained from seven randomly chosen locations within the sample. The curves were recorded during application of a maximal loading force of 1.0 nN at a speed of 0.25 µm/s. Curves that deviated from the established mathematical model (*i.e.*, unbiased) were discarded.

Endothelial Cell Culture Experiments

Human umbilical vein endothelial cells (HUVECs, CC-2519, Lonza) were cultured in complete VascuLife® EnGS medium (LL-0002, Lifeline Cell Technologies) supplemented with EGM^{TM} medium (CC-3124, Lonza) and 2% fetal bovine serum. HUVECs (5x10^4 cells/well; passaged 5/6) were seeded onto 96-well plates and cultured at 37°C under 5% CO_2 for 24 hours. Then, 100 mU/mL neuraminidase (N2876, Sigma), with or without a cocktail of glycocalyx precursors in reduced fetal bovine serum (0.5%) cell media, was used to treat cells for 48 hours. We have previously shown that this concentration of neuraminidase is an effective approach to cause glycocalyx destruction (152). To prepare the cocktail of glycocalyx precursors, a mortar and pestle was used to grind the content of the capsules to a fine powder, and 0.2 mg/mL was solvent extracted in 95% EtOH. The soluble extract was diluted 1:4000 into cell media. After treatments, cells were fixed in 4% paraformaldehyde (15710, Electron Microscopy Sciences) prior to immunofluorescence staining and imaging. To evaluate the extent of glycocalyx coverage on endothelial cells, fixed cells were washed twice with phosphate-buffered saline and incubated for 30 minutes with 3 mg/mL Alexa-488-conjugated wheat-germ agglutinin (WGA-Alexa-555, W32464, Molecular Probes). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, No. D9542, Sigma-Aldrich, 1:500 dilution). A microplate reader (BioTeK Synergy H1, Agilent, Santa Clara, CA) equipped with area scanning mode was used to collect fluorescence measurements. Excitation/Emission wavelengths were set at 539/580 nm for WGA and 377/477 nm for DAPI. The WGA signal was normalized to DAPI fluorescence to control for variations in cell number per well.

Clinical Trial

All human study procedures conformed to the Declaration of Helsinki and were approved by the University of Missouri Institutional Review Board (protocol 2062542), the R&D committee at the Harry S Truman VA, and the Med/Surg VA Data Monitoring Committee. The double-blinded, randomized, placebo-controlled clinical trial was registered at ClinicalTrials.gov (NCT05205005) with an FDA IND 164629. Written informed consent was obtained from all subjects before any study procedures. Men (n=23) and women (n=1) Veterans with a diagnosis of T2D, 45 to 74 years of age, overweight or obesity (BMI 25-45 kg/m^2), an HbA1c <9%, and fasting blood glucose <200 mg/dL at screening visit were recruited from the Harry S Truman VA Hospital. Participants who, within the past year, had experienced a cardiovascular event or had uncontrolled hypertension (blood pressure exceeding 180 mmHg systolic or 110 mmHg diastolic) were excluded. Additionally, anyone with stage IV or V chronic kidney disease (GFR <30 mL/min) or liver disease, active cancer, or on immunosuppressant medications were excluded. Furthermore, excessive alcohol consumption (more than 14 drinks per week for men or seven drinks per week for women), current use of hormone replacement therapy, or uncontrolled thyroid dysfunction (indicated by abnormal TSH levels within three months of enrollment) disqualified potential participants. Individuals with difficulty swallowing capsules, allergies to any supplement ingredients (including glucosamine extract, fucoidan extract, olive extract, artichoke extract, red and white grapes extract, melon concentrate, and hyaluronic acid), or pregnant women or with plans to become pregnant during the study were also excluded.

Study Procedures

Participants were randomized 1:1 to either DSGP or placebo treatment (**Figure 3.1**). Preparation and administration of the supplement/placebo capsules were directed by the VA Hospital Investigational Pharmacy. All members of the investigative team involved in data acquisition and analysis were blinded to group assignment, and participants were not informed of their group status. Following randomization and after baseline study visits, participants were provided DSGP supplementation in the form of Endocalyx® (Microvascular Health Solutions, Alpine, UT) or a matching placebo (Nu-Mag, 10 mg; rice flour, 830 mg; and magnesium stearate veggie powder, 10 mg). Participants were instructed to take three capsules of DSGP twice daily (3,712.5 mg/day), preferably with breakfast and dinner, or a placebo for eight weeks. One capsule contained fucoidan extracted from *Laminaria japonica* (106.25 mg), glucosamine sulphate (375.0 mg), hyaluronic acid (17.5 mg), and a blend of superoxide dismutase and polyphenols (120.0 mg). Adherence was measured with a capsule count. The total number of capsules ingested was calculated as the number of capsules provided minus the number of capsules returned. Percent adherence was determined as the ratio of capsules ingested to total capsules provided x 100. All study participants except one in the placebo group returned study capsules on their final study visit. The DSGP dosage for this study was determined through a conversion of pre-clinical results in diabetic mice, which received 100 mg/kg/day of DSGP for four weeks. Following eight weeks of supplementation, participants returned to the laboratory for final study visit measurements. In preparation for the study visits, participants were asked to fast overnight (at least eight hours), abstain from tobacco and caffeine intake the morning of the visit, and avoid exercise and alcohol consumption for at least 24 hours prior. Furthermore, participants were asked to withhold their diabetes medications the morning of their visit. Participants were also instructed to maintain their usual diet during the intervention.

24-hour Ambulatory Blood Pressure Monitoring

At least 24 hours prior, and within seven days of their baseline and final study visits, participants were fitted with a 24-hour ambulatory blood pressure monitor (ABPM 7100, Baxter International, Deerfield, IL) cuff applied to the dominant upper arm by a trained technician. The ABPM devices were programmed to follow a specific schedule for blood pressure readings over a 24-hour period. During waking hours, the devices were set to take a reading every 30 minutes. For sleeping hours, the readings were programmed to occur at 60-

minute intervals. In case a reading failed on the first try, the devices were designed to automatically reattempt the measurement within two minutes. Participants were instructed to maintain their standard lifestyle patterns. Participants returned the ABPM device at the time of the baseline, and final visits (**Figure 3.1**), and data were downloaded and analyzed with technical software (CardioPerfect WorkStation) (175). The circumference of the upper arm was measured via a tape measure to determine the appropriate cuff size. Different cuff sizes ranging from small to extra-large adult were used to accommodate a diverse sample.

Assessment of Carotid-to-Femoral Pulse Wave Velocity and FMD

Carotid-to-femoral pulse wave velocity (cfPWV) was measured using the SphygmoCor XCEL system (AtCor Medical, Itasca, IL) with a cuff to assess aortic stiffness, as described previously (24). For assessment of brachial artery FMD, a two-dimensional/Doppler ultrasound (GE Logiq P5) was used, following prior methods and published guidelines (24, 176). Briefly, an 11-MHz linear array transducer was secured with a clamp and placed over the brachial artery. A rapid inflating cuff (Hokanson) was placed on the forearm ~5 cm distal to the antecubital fossa. In duplex mode, using a pulsed frequency of 5 MHz and corrected for a 60° insonation angle, simultaneous diameter and velocity signals were obtained. The sample volume was adjusted to encompass the entire vessel lumen without extending beyond the walls, and the cursor was set at mid-vessel. Real-time capture software (Elgato Video Capture, Elgato, CA) was used to record two minutes of ultrasound imaging and then the cuff was inflated to a pressure of 250 mmHg for five minutes. Three minutes of ultrasound imaging were collected following cuff deflation. Next, as previously described (177), patients were prepared for superficial femoral artery FMD assessment. Specialized edge-detection software (Cardiovascular Suites 4, Quipu srl, Pisa, Italy) was used for offline analysis of recorded vascular variables. FMD percent change was calculated as follows:

$$\mathrm{FMD}\ (\%) = 100 \times \frac{D_{peak} - D_{bl}}{D_{bl}},$$

where D_{peak} and D_{bl} are the peak and baseline diameters reported in mm. The shear rate was estimated as:

$$\gamma = \frac{4v}{D},$$

where γ is the shear rate reported in s^{-1}, v is mean blood velocity in cm/s, and D is the diameter in cm.

In Vivo Glycocalyx Thickness

The integrity of the endothelial glycocalyx was assessed via bedside intravital microscopy using a side-stream dark field camera (CapiScope HVCS, KK Technology, Honiton, UK) to visualize the sublingual microvasculature, as previously described (64). Briefly, side-stream dark field video-microscopy detects hemoglobin found in moving red blood cells (RBC) within the sublingual microcirculation via green light emitting stroboscopic diodes at a wavelength of 540 nm. Image acquisition and analysis are automated using a GlycoCheck™

system (MicroVascular Health Solutions, Alpine, UT) based on predefined criteria for motion, intensity, and focus. Following analysis, the thickness of the endothelial glycocalyx in microvessels with internal diameters ranging from 4 to 25 μm is reported as perfused boundary region (PBR). PBR represents the distance between flowing RBC and the physical width of the negatively charged glycan structures (glycosaminoglycans and sialic acid) that make up the glycocalyx. A larger PBR value indicates deeper penetration of RBC into the glycocalyx, signifying its degradation and thinning (155). Penetration of RBC into the glycocalyx can be velocity-dependent, thus, to minimize possible flow-dependent variability in PBR estimation, $PBR_{dynamic}$ was used (178). Microvascular vessel density was obtained from the number of measurements sites, with each microvessel measurement site representing 10 μm of length (64). Cumulative microvessel length was divided by the total area recorded to calculate microvascular vessel density. The absolute value for measuring capillary blood volume (CBV_{abs}) was determined from the number of capillary segments multiplied by the capillary segment length and the segment-specific capillary cross-sectional area. This value was then multiplied with the CBV ratio which accounts for the average RBC velocity (V_{RBC}) in larger blood vessels versus small capillaries ($= V_{RBC}(D \geq 10 \mu m)/V_{RBC}(D \leq 7 \mu m)$). An increase in CBV relative to larger vessel blood volume will increase the CBV ratio. Multiplying CBV_{abs} with the CBV ratio gives a static CBV (CBV_{static}) value. To take the recruitment capabilities of additional capillaries into account, capillary recruitment (CR) was estimated by measuring the slope of the relationship between V_{RBC}

($D \leq 7$ μm) and V_{RBC} ($D \geq 10$ μm). Multiplying CBV_{static} by (1+CR) calculates dynamic CBV ($CBV_{dynamic}$) (178). Lastly, CBV and PBR proportions can change inversely, thus, the quotient of CBV/PBR was used to calculate one overall dynamic microvascular health score ($MVHS_{dynamic}$) (178).

Assessment of Leg Blood Flow, Total Peripheral Resistance (TPR), and Skeletal Muscle Microvascular Perfusion in Response to Insulin

Insulin stimulated-leg blood flow was assessed as another indicator of vascular function and as described previously (24). Briefly, intravenous catheters were inserted in both antecubital veins for blood sampling and infusions of insulin and dextrose. Approximately 20 minutes after catheter placement, assessments of blood flow in the superficial femoral artery followed by microvascular perfusion in the quadriceps muscle (vastus lateralis) were obtained via a two-dimensional/Doppler ultrasound and contrast-enhanced ultrasound, respectively (24). Then, insulin (Novolin-R U-100) was prepared via dilution in 250 mL of 0.9% saline along with 5 mL of whole blood obtained from the subject to a final concentration of 500 mU/mL. Insulin was then infused at a constant dosage of 80 mU/m^2 body surface area/min. Following the 60-minute infusion, superficial femoral artery blood flow and quadriceps muscle microvascular perfusion were reassessed. Total peripheral resistance (TPR) was assessed noninvasively using finger photoplethysmography (Human NIBP, ADInstruments) and calibrated to upper arm sphygmomanometry. Arterial blood pressure waveforms using the Modelflow method (LabChart, ADInstruments), which incorporates sex and age, were used for estimation of TPR. Whole blood glucose was measured every five minutes and maintained at fasted levels. This was achieved by variable infusion rates of a 20% dextrose solution. Plasma was obtained and stored at −80°C for later biochemical analysis.

Measurement of Fasting Plasma Nitrite and Biochemical Parameters

Plasma nitrite concentrations, a surrogate of NO, were assessed using an ozone-based reductive chemiluminescence NO analyzer (CLD88, Eco Physics) according to the manufacturer guidelines and previously described methods (179, 180). Plasma samples (100 µL) were injected into a purge vessel containing 3.5mL of glacial acetic acid and 0.5mL ascorbic acid (0.5 mM) (181, 182), which was then purged with pure nitrogen in-line with the CLD88 gas-phase NO analyzer. The chemiluminescence signal was acquired using eDAQ ChartTM v5.5.27 software, and NO quantification was performed using the flow injection analysis (FIA) software extension (ADInstruments, Australia). The FIA software calculated the area under the curve for each sample peak, which was then converted to a concentration using a calibrated standard curve generated with known sodium nitrite standards. Plasma samples obtained during the insulin infusion were assessed for insulin concentrations using a commercially available kit (ALPCO Cat. No. 80-INSHU-E10.1, Salem, NH). Concentrations of Endothelin-1 (#EIAET1, Invitrogen, Thermo Fisher Scientific), Glucose (#10009582, Glucose Colorimetric Assay, Caymen Chemical), Glypican-1 (#ab270217, SimpleStep, Abcam), and Hyaluronan (#DHYAL0, Quantikine, Bio-

Techne) in plasma were assessed using commercially available ELISA kits following manufacturer instructions.

Statistical Analysis

All power analyses used an α of 0.05 and 80% power for the clinical trial. We determined that a sample size of 10 subjects per group would provide 82% power and be sufficient to detect significant differences in outcomes based on pre-clinical data and preliminary unpublished data in humans from our lab. We enrolled 24 subjects to account for any potential dropouts. Data are presented as mean ± standard error of the mean (SEM). Shapiro-Wilk Test was performed for the assessment of data distribution. When data were not normally distributed, nonparametric tests, Mann-Whitney U (Wilcoxon rank-sum test) and Wilcoxon signed-rank tests, were performed. Treatment-related differences in outcomes were determined using two-tailed paired and unpaired Student's t-test and unpaired one- and two-way repeated-measures ANOVA, as appropriate. Bonferroni post-hoc tests were performed when significant interactions were found. The results were considered significant when $P<0.05$. Statistical analyses were performed using GraphPad Prism (version 10.0) while the investigative team remained blinded to treatment groups.

RESULTS

As depicted in **Figure 3.2A**, vehicle-treated db/db mice exhibited a reduced endothelial glycocalyx length compared with db/+ control mice ($P<0.05$), as assessed via AFM in en face aortic explants. This deficit was restored in db/db mice treated with DSGP for four weeks ($P<0.05$). Glycocalyx stiffness and cell cortical stiffness were also higher in vehicle-treated db/db mice, relative to db/+ controls ($P<0.05$), and again normalized by DSGP treatment ($P<0.05$). As shown in **Figure 3.2B**, FMD in isolated mesenteric arteries was reduced in vehicle-treated db/db mice compared with db/+ controls ($P<0.05$), indicative of impaired shear stress mechanotransduction. This impairment was corrected with DSGP treatment ($P<0.05$). No group differences were noted in vasodilatory responses to SNP ($P>0.05$). The incremental modulus of elasticity, a measurement of structural stiffness, was elevated in vehicle-treated ($P<0.05$) but not DSGP-treated db/db mice ($P>0.05$). As displayed in **Figure 3.2C**, vehicle-treated db/db mice exhibited impaired ACh-induced relaxation in aortic rings, relative to db/+ controls ($P<0.05$). This impairment was corrected with DSGP treatment ($P<0.05$). No group differences were noted in relaxation responses to SNP ($P>0.05$). Lastly, as shown in **Figure 3.2D**, cultured endothelial cells treated with the cocktail of glycocalyx precursors for 48 hours displayed greater glycocalyx coverage, as assessed using WGA staining, compared with vehicle-treated cells ($P<0.05$).

A total of 24 Veterans with T2D were randomized to either DSGP or placebo treatment. Two subjects allocated to DSGP withdrew from the study, leaving 10 and 12 subjects for analysis (**Figure 3.3**). Reasons for exclusion are also summarized in **Figure 3.3**. **Table 3.1** summarizes subject characteristics, anthropometrics, and blood profile parameters before and after DSGP vs.

placebo treatment for eight weeks. A significant interaction effect was observed for both HOMA-IR and fasting insulin (*P*<0.05); however, the Bonferroni post hoc test did not reveal any significant differences for either outcome (*P*>0.05). DSGP did not have a significant impact on any of the other parameters assessed (*P*>0.05). Also, in contrast to the findings in mice, DSGP did not have an effect on vascular outcomes in this cohort of subjects with T2D (*P*>0.05). Indeed, as depicted in **Figure 3.4A**, the delta change in PBR from pre- to post-intervention was not different between placebo and DSGP-treated subjects (*P*>0.05). Similarly, relative to placebo, DSGP did not affect brachial and femoral artery FMD (*P*>0.05, **Figure 3.4B**), plasma nitrite (*P*>0.05, **Figure 3.4C**), cfPWV (*P*>0.05, **Figure 3.4D**), or 24-hour average mean arterial pressure (*P*>0.05, **Figure 3.4E**). We also assessed leg blood flow, leg vascular conductance, skeletal muscle perfusion, and total peripheral resistance in response to systemic insulin infusion, and no improvements were observed after DSGP treatment (*P*>0.05, **Figure 3.4F**). **Table 3.2** depicts additional cardiovascular and hemodynamic information.

DISCUSSION

The primary findings of this investigation are that DSGP increases endothelial glycocalyx length, improves FMD, and reduces arterial stiffness in db/db mice, a rodent model of T2D. However, these observations are not translatable to individuals with T2D. Indeed, eight weeks of DSGP did not enhance the endothelial glycocalyx, nor improve any indices of vascular function in Veterans with T2D.

The impetus for this work stems from the increasing evidence that degradation of the endothelial glycocalyx, a phenomenon well documented in T2D (60, 61, 65, 70, 142, 152, 168–170), contributes to endothelial dysfunction and, consequently, the development of CVD (67). One of the key functions of the endothelial glycocalyx is to serve as a mechanosensor of shear stress (66). A plethora of studies, both *in vitro* and *in vivo*, demonstrate shear stress is a crucial mechanical signal to the vascular endothelium that promotes the production of nitric oxide and other vascular protective molecules (183, 184). As such, impaired shear stress mechanotransduction, caused in part by the destruction of glycocalyx structures, is considered an important contributor to endothelial dysfunction and cardiovascular complications. This positions the endothelial glycocalyx as an attractive vascular component for intervention, particularly in disease states such as T2D, where degradation of the endothelial glycocalyx is common. Certainly, therapeutic strategies designed to restore the endothelial glycocalyx could have a significant positive impact on the prevention and treatment of T2D-associated CVD.

After surveying available strategies to modify the glycocalyx length with translational potential, efforts were first directed toward targeting neuraminidase. This enzyme cleaves sialic acid from glycocalyx structures, and it is increased in the circulation of individuals with T2D (152, 185). Recently, we corroborated in cultured endothelial cells that neuraminidase exposure diminishes glycocalyx coverage and that, in isolated arteries, this is accompanied by impaired FMD, indicative of impaired shear stress mechanotransduction (152). Subsequently, it was shown that inhalation of the FDA-approved neuraminidase inhibitor, Zanamivir, reduced plasma neuraminidase activity, enhanced endothelial glycocalyx length, and improved FMD in db/db mice (152). However, these attempts to translate these findings to humans failed. Indeed, Zanamivir inhalation following the FDA-approved dosing regimen did not reduce plasma neuraminidase activity nor improve glycocalyx length and FMD in individuals with T2D, likely due to the limited concentration of plasma Zanamivir achieved by such dosing (152). These negative findings in humans led to the consideration of DSGP as another potential strategy to restore the endothelial glycocalyx in T2D.

The focus on DSGP was founded partly on the preclinical work from Machin *et al*. (68), demonstrating that feeding aged mice with a cocktail of glycocalyx precursors available as a dietary supplement enhanced glycocalyx thickness and improved vascular function. As an initial step, an endothelial cell culture model of glycocalyx degradation was utilized, which consists of endothelial exposure to neuraminidase (152). Treatment of glycocalyx-degraded endothelial cells with the cocktail of glycocalyx precursors, which contains glucosamine sulfate, fucoidan, superoxide dismutase, and high molecular weight hyaluronan, promoted glycocalyx growth. Next, treatment of db/db mice with DSGP for four weeks, similar to findings in aged mice (68), augmented endothelial glycocalyx thickness (i.e., length) as assessed via AFM in aortic explants. The increase in glycocalyx length with DSGP was associated with reduced glycocalyx stiffness and cellular stiffness. This finding is of interest as data are available suggesting that a stiff glycocalyx may compromise its ability to transmit mechanical forces into the cell (61, 186). Along these lines, DSGP improved FMD in isolated mesenteric arteries without influencing endothelium-independent dilation to SNP. Further, the improvement in endothelial function was accompanied by a reduction in arterial stiffness.

These overall positive results in mice led for the next logical step; that is, assess the efficacy of DSGP in patients with T2D using a randomized controlled trial. In contrast to the hypothesis, DSGP for eight weeks did not enhance the integrity of the endothelial glycocalyx, as determined by the PBR assessed in the sublingual circulation using the GlycoCheck, nor improve indices of vascular function in Veterans with T2D. This pilot study, while small in sample size, involved multiple vascular outcomes, including FMD in the brachial and superficial femoral arteries, insulin-stimulated leg blood flow and skeletal muscle perfusion via Doppler and contrast-enhanced ultrasound, and arterial stiffness as assessed using cfPWV. Despite the comprehensive vascular phenotyping, the results consistently demonstrated a lack of an effect of DSGP. Likewise, the intervention did not beneficially impact body composition, metabolic outcomes, or lipid profiles. Neither resting (**Table 3.1**) nor 24-hour ambulatory blood pressure (**Table 3.2**) were affected by DSGP. Of note, and as shown in **Table 3.1**, subjects randomly assigned to placebo tended to have a lower systolic blood pressure compared to those assigned to DSGP (*P*=0.08). While speculative, this may be attributable to the fact that, by chance, more subjects assigned to placebo were using SGLT2 inhibitors and GLP-1 receptor agonists, agents with documented blood pressure-lowering effects (159, 160).

Coincidently, during the preparation of this manuscript, the article by Gimblet *et al*. (69) was published demonstrating, in a similar size randomized controlled trial (n=23), that 3,712.5 mg/day of DSGP for 12 weeks failed to enhance glycocalyx integrity (also based on the measurement of PBR using the GlycoCheck^{TM}), improve brachial artery FMD, or reduce cfPWV in older adults. The similarity in negative findings between the two simultaneously and independently conducted clinical trials are a strength in that it reinforces the notion that this glycocalyx-targeted therapy is not efficacious for improving human vascular function. The findings of this work, together with findings by Gimblet *et al*. (69) and another previously published clinical trial using the neuraminidase inhibitor Zanamivir (152), show that restoring the endothelial glycocalyx in humans remains an unresolved challenge. However, this is not a universal finding. A recently published manuscript by van der Velden *et al.* (70) demonstrated in a cohort of South-Asian Surinamese descent individuals with T2D that 2,475 mg/day of DSGP for 12 weeks reduced PBR, indicative of increased endothelial glycocalyx integrity. While there is not a strong argument for explaining this discrepancy in results, it could be speculated that differences could be attributed to the fact that the study participants in this clinical trial had a higher usage rate of antidiabetic agents with known cardiovascular benefits.

Several aspects of the present investigation warrant further consideration. First, while the VA clinical trial was open for recruitment to both sexes, the vast majority of participants were men. This was partly because approximately 80% of patients at the VA are males, and no specific recruitment strategies were put in place to maintain an equal distribution of men and women, which can be considered a weakness of this pilot study. However, it should be noted that female mice were used for the preclinical studies. Second, in the clinical study, the PBR-based assessment of endothelial glycocalyx dimensions was performed in the sublingual circulation following established procedures (63). No other methods are available for directly assessing glycocalyx integrity in humans. At this time, it cannot be attested that an index of glycocalyx integrity in the sublingual circulation indicates glycocalyx integrity in other vascular beds. However, it is reasonable to infer that if the intervention is systemic (as is the case with DSGP), an improvement in glycocalyx integrity in one vascular bed should reflect improvements in glycocalyx integrity in all beds, even if the magnitude of change is variable. Lastly, Endocalyx®, the patented and commercially available dietary supplement used in this study, contains multiple ingredients designed to synthesize, repair, and provide antioxidant support to the endothelial glycocalyx (187). Additional studies could be conducted to study the vascular effects of the different ingredients in isolation using *in vitro* and/or *in vivo* models.

In aggregate, the findings presented herein conceptually support the notion that restoration of the endothelial glycocalyx using DSGP can lead to improvements in vascular function in a mouse model of T2D. However, DSGP as a therapeutic strategy to enhance vascular function does not appear to be efficacious in Veterans with T2D, and based on recent data from others, it is likely not efficacious for many other populations. Ideally, this sum of work stimulates more innovative ideas for therapeutic strategies to effectively boost the endothelial glycocalyx in T2D and other disease states.

GRANTS

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DISCLOSURES

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

Figure 3.1. Experimental design of clinical trial. Following randomization to either dietary supplementation of glycocalyx precursors (DSGP) or placebo treatment, and at least 24 hours prior, but within seven days of their baseline and final study visits, participants were fitted with a 24-hour ambulatory blood pressure monitor for blood pressure assessments.

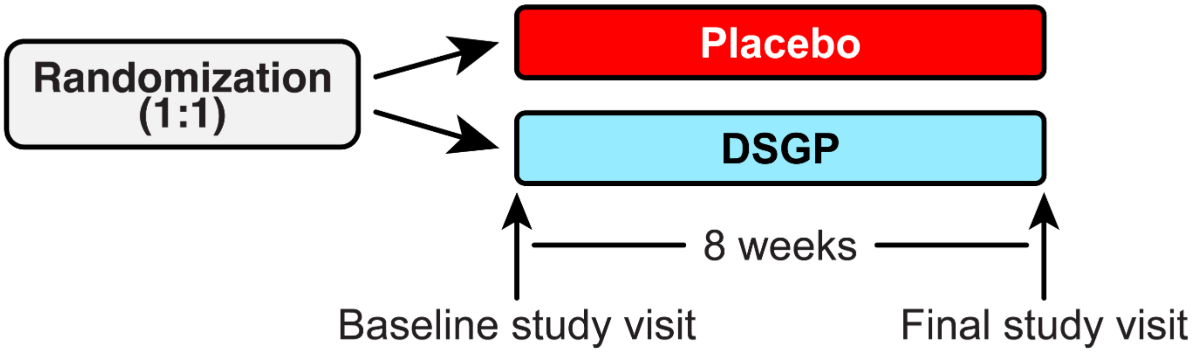

Figure 3.2. Vascular effects of dietary supplementation of glycocalyx precursors (DSGP) vs. vehicle (Veh) treatment in db/db mice, and the effect of glycocalyx precursors on glycocalyx coverage in cultured endothelial cells. **A)** Endothelial glycocalyx length, as well as glycocalyx and cell cortical stiffness, was assessed in en face aortic explants (schematic representation shown in the inset) using atomic force microscopy in db/+ control mice and in db/db mice treated with DSGP vs. vehicle for four weeks. Statistical analysis was performed using unpaired one-way ANOVA with Bonferroni post hoc test; n=9–20/group. **B)** Flow-mediated dilation (FMD) and sodium nitroprusside (SNP)-induced dilation in isolated mesenteric arteries. Data are expressed as percentages of dilation from phenylephrine preconstriction in response to increasing flow rates and SNP concentrations. Mesenteric artery stiffness was assessed via the incremental modulus of elasticity (Einc) at increasing intraluminal pressures under calcium-free conditions. Statistical analysis was performed using unpaired two-way ANOVA with repeated measures and Bonferroni post hoc test; n=8-9/group. **C)** Aortic relaxation was assessed in response to increasing concentrations of acetylcholine (ACh) and SNP following preconstriction with U46619. Statistical analysis was performed using unpaired two-way ANOVA with repeated measures and Bonferroni post hoc test; n=6–16/group. **D)** Human umbilical vein endothelial cells exposed to neuraminidase to induce glycocalyx degradation were treated with vehicle vs. the cocktail of glycocalyx precursors (GP) for 48 hours. Glycocalyx was determined by staining with fluorescent wheat germ agglutinin (WGA) and fluorescence intensity quantification. Statistical analysis was performed using unpaired Student's t-tests; n=11-12/condition. Data are presented as means ± SEM. Individual data points are also presented as appropriate. *$P<0.05$ vs. db/+, #$P<0.05$ vs. db/db+Veh or Veh.

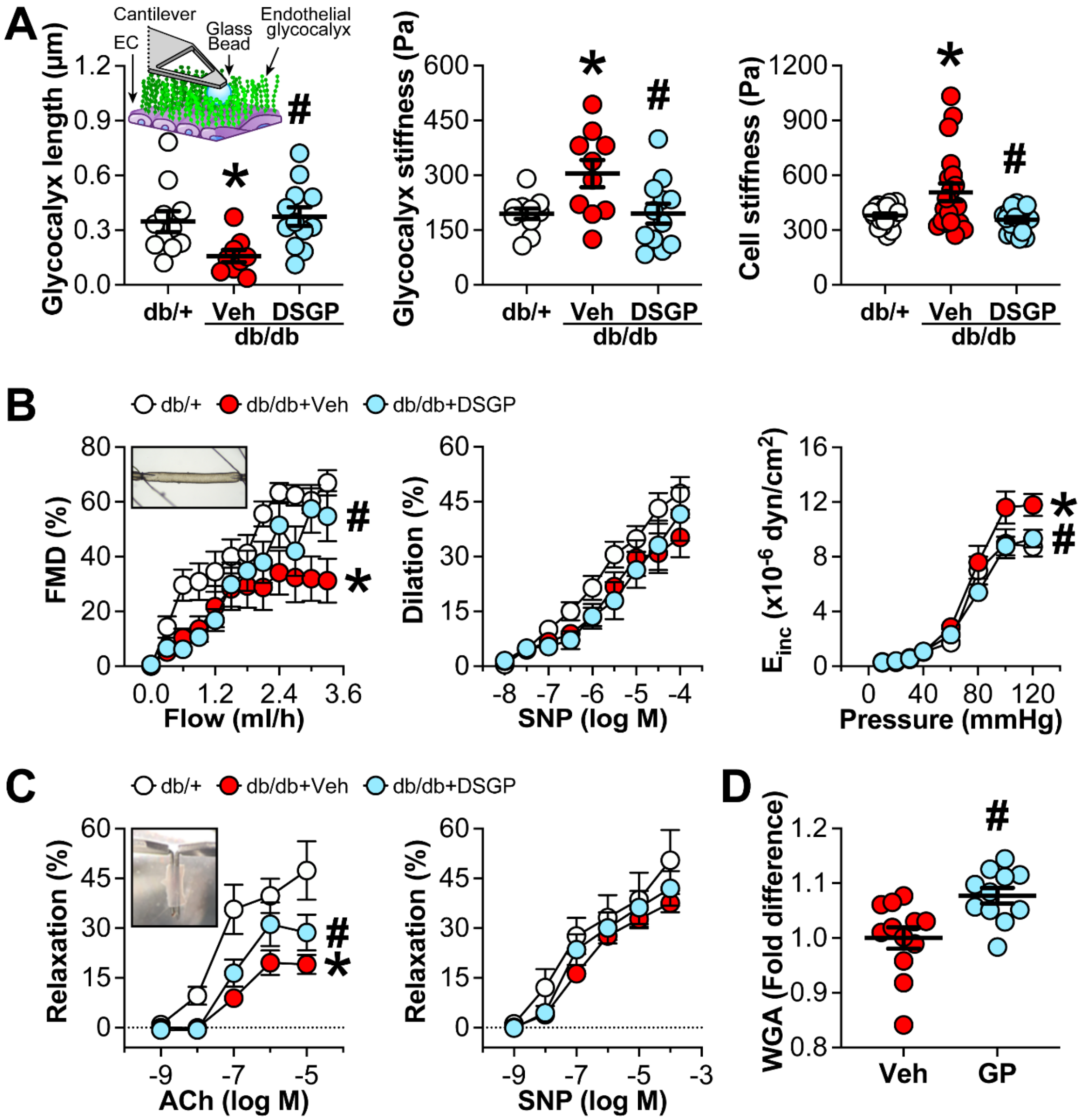
A
Cantilever
EC
Glass Bead
Endothelial glycocalyx
Glycocalyx length (µm)
1.2
0.9
0.6
0.3
0.0
db/+
Veh
DSGP
db/db
*
#
Glycocalyx stiffness (Pa)
600
450
300
150
0
db/+
Veh
DSGP
db/db
*
#
Cell stiffness (Pa)
1200
900
600
300
0
db/+
Veh
DSGP
db/db
*
#
B
db/+
db/db+Veh
db/db+DSGP
FMD (%)
80
60
40
20
0
0.0
1.2
2.4
3.6
Flow (ml/h)
#
*
Dilation (%)
60
45
30
15
0
-8
-7
-6
-5
-4
SNP (log M)
E_{inc} (x10^{-6} dyn/cm^2)
16
12
8
4
0
0
40
80
120
Pressure (mmHg)
*
#
C
db/+
db/db+Veh
db/db+DSGP
Relaxation (%)
60
45
30
15
0
-9
-7
-5
ACh (log M)
#
*
Relaxation (%)
60
45
30
15
0
-9
-7
-5
-3
SNP (log M)
D
WGA (Fold difference)
1.2
1.1
1.0
0.9
0.8
Veh
GP
#

Figure 3.3. Flow diagram for the clinical trial.

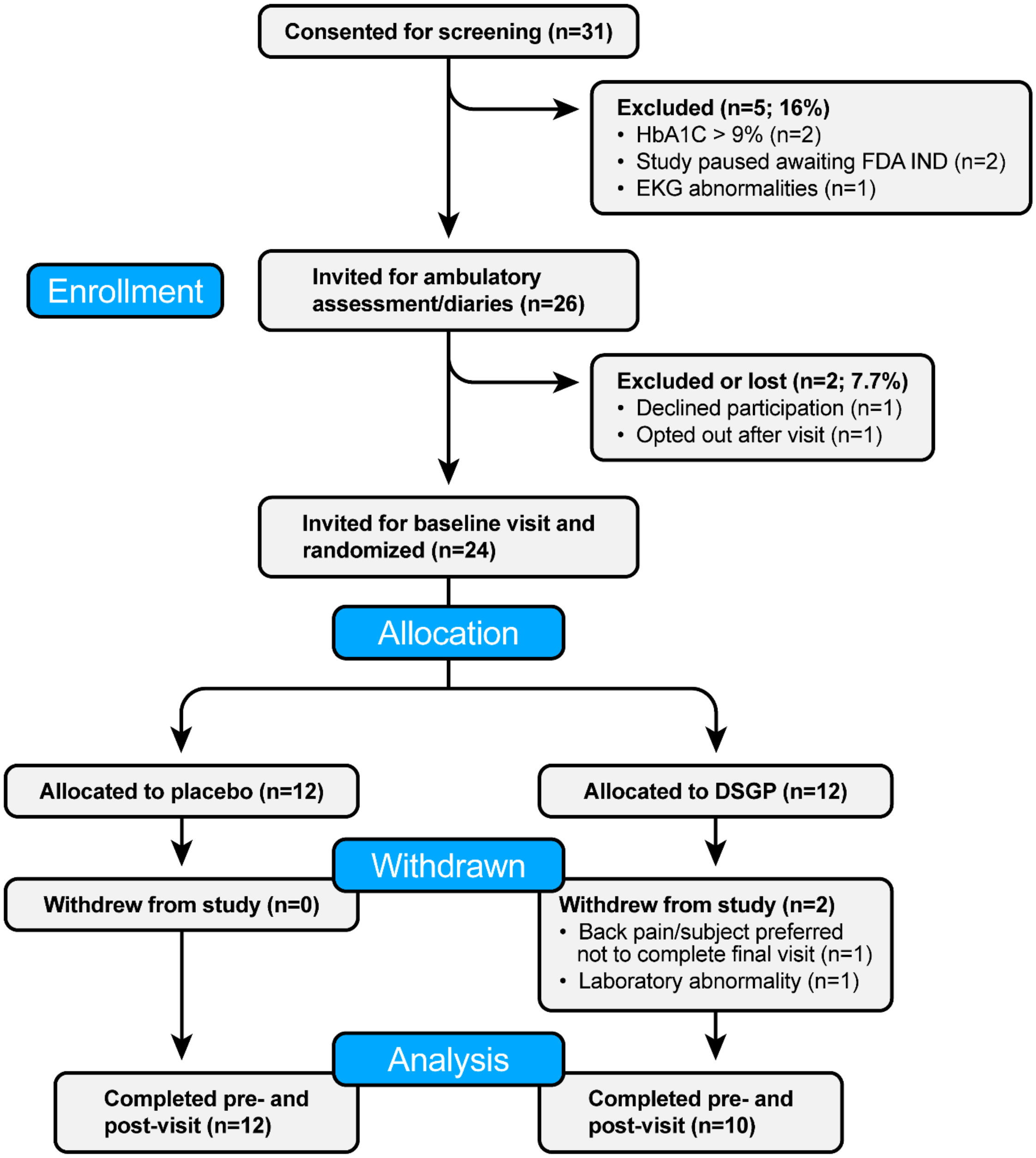

Figure 3.4. Vascular effects of dietary supplementation of glycocalyx precursors (DSGP) vs. placebo treatment in Veterans with type 2 diabetes. **A)** Delta change in perfused boundary region (PBR) from pre- to post-intervention. Statistical analysis was performed using unpaired Student's t-tests; n=10-12/group. **B)** Delta change in brachial and femoral artery flow-mediated dilation (FMD) from pre- to post-intervention. Statistical analysis was performed using unpaired Student's t-tests; n=10-12/group. **C)** Delta change in plasma nitrite from pre- to post-intervention. Statistical analysis was performed using unpaired Student's t-tests; n=10-12/group. **D)** Delta change in carotid-to-femoral pulse wave velocity (cfPWV) from pre- to post-intervention. Statistical analysis was performed using unpaired Student's t-tests; n=8-11/group. **E)** Delta change in 24-hour average mean arterial blood pressure (MAP) from pre- to post-intervention. Statistical analysis was performed using unpaired Student's t-tests; n=9-11/group. **F)** Delta change in leg blood flow and vascular conductance, skeletal muscle (SkM) perfusion, and total peripheral resistance (TPR) response to insulin from pre- to post-intervention. Measurements were performed at baseline and at 60 minutes of systemic insulin infusion (with the coinfusion of dextrose to maintain euglycemia). The difference between time points was then calculated to capture the insulin response as the outcome variable. Statistical analysis of normally distributed data (SkM perfusion) was performed using unpaired Student's t-tests. Non-normally distributed data (leg blood flow, vascular conductance, and TPR) were analyzed using unpaired Mann–Whitney U (Wilcoxon rank-sum) test; n=9-12/group. Data are presented as means ± SEM. Individual data points are also presented. *P* values for all comparisons are shown.

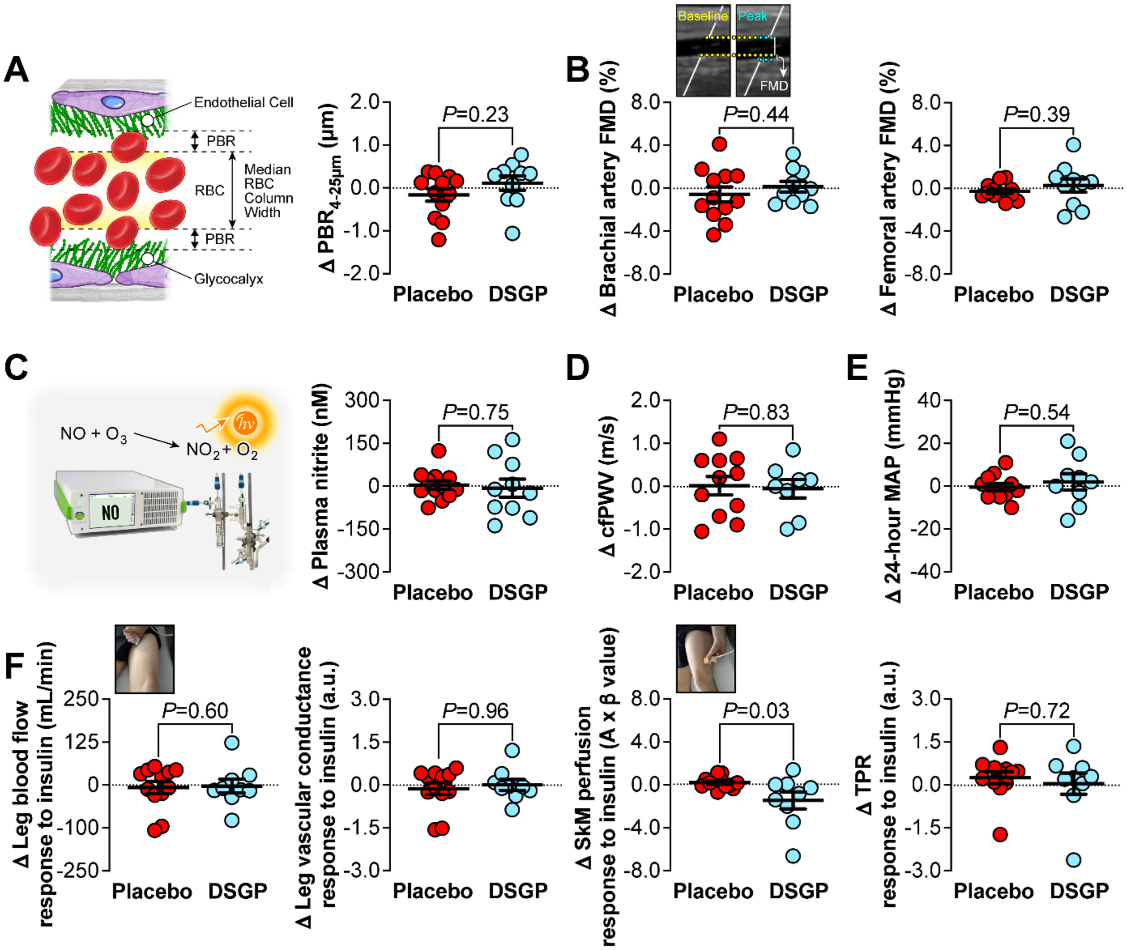
A
Endothelial Cell
PBR
RBC
Median RBC Column Width
PBR
Glycocalyx
Δ PBR4-25μm (μm)
2.0
1.0
0.0
-1.0
-2.0
P=0.23
Placebo
DSGP
B
Baseline
Peak
FMD
Δ Brachial artery FMD (%)
8.0
4.0
0.0
-4.0
-8.0
P=0.44
Placebo
DSGP
Δ Femoral artery FMD (%)
8.0
4.0
0.0
-4.0
-8.0
P=0.39
Placebo
DSGP
C
NO + O3
hν
NO2 + O2
NO
Δ Plasma nitrite (nM)
300
150
0
-150
-300
P=0.75
Placebo
DSGP
D
Δ cfPWV (m/s)
2.0
1.0
0.0
-1.0
-2.0
P=0.83
Placebo
DSGP
E
Δ 24-hour MAP (mmHg)
40
20
0
-20
-40
P=0.54
Placebo
DSGP
F
Δ Leg blood flow response to insulin (mL/min)
250
125
0
-100
-250
P=0.60
Placebo
DSGP
Δ Leg vascular conductance response to insulin (a.u.)
3.0
1.5
0.0
-1.5
-3.0
P=0.96
Placebo
DSGP
Δ SkM perfusion response to insulin (A x β value)
8.0
4.0
0.0
-4.0
-8.0
P=0.03
Placebo
DSGP
Δ TPR response to insulin (a.u.)
3.0
1.5
0.0
-1.5
-3.0
P=0.72
Placebo
DSGP

Table 3.1. Subject characteristics, anthropometrics, and blood profile parameters before and after dietary supplementation of glycocalyx precursors (DSGP) vs. placebo treatment in Veterans with type 2 diabetes. *$P<0.05$ vs. Before. Data are presented as means ± SEM.

	Placebo Mean ± SE (n=12)		**DSGP Mean ± SE (n=10)**	
Race (n, %)				
Caucasian/Non-Hispanic	(12, 100%)		(10, 100%)	
	Before	**After**	**Before**	**After**
Age (years)	61 ± 2	-	66 ± 2	-
Length of T2D diagnosis (years)	13 ± 2	-	15 ± 3	-
Capsule consumption adherence (%)	97.3 ± 0.01	-	97.9 ± 0.01	-
Height (cm)	176 ± 2	-	175 ± 2	-
Body weight (kg)	107.4 ± 4.2	106.8 ± 4.3	103.1 ± 6.0	103.7 ± 5.6
Body mass index (kg/m^2)	35 ± 1.2	34 ± 1.2	34 ± 1.6	34 ± 1.6
Body fat mass (%)	40 ± 2	39 ± 2	38 ± 2	39 ± 2*
Lean body mass (kg/m^2)	19.7 ± 0.5	19.9 ± 0.4	20.0 ± 0.8	20.2 ± 0.8
Systolic BP (mmHg)	130 ± 3	129 ± 5	143 ± 6	144 ± 8
Diastolic BP (mmHg)	78 ± 2	78 ± 2	80 ± 2	82 ± 2
Heart rate (bpm)	67 ± 2	66 ± 3	59 ± 2	61 ± 2
Fasted blood glucose (mg/dL)	134 ± 9	131 ± 8	127 ± 16	135 ± 14
Fasted insulin (uU/mL)	12.4 ± 1.9	9.4 ± 1.5	12.3 ± 3.5	14.7 ± 4.3
HOMA-IR (mg/dL)	4.0 ± 0.6	3.0 ± 0.5	3.7 ± 1.1	4.6 ± 1.2
Hemoglobin A1C (%)	7.5 ± 0.2	-	7.0 ± 0.3	-
Estimated glomerular filtration rate (mL/min)	86.6 ± 6.1	-	77.7 ± 8.2	-
Glypican-1 (pg/mL)	14491 ± 879	14175 ± 853	15876 ± 1133	15475 ± 714
Hyaluronan (ng/mL)	41 ± 8	40 ± 6	47 ± 8	47 ± 8

Endothelin-1 (pg/mL)	64 ± 5	66 ± 4	65 ± 5	63 ± 8
Nitrite (nM)	62 ± 13	66 ± 15	96 ± 18	89 ± 27
Medications				
Biguanide	10		7	
Glucagon-like peptide-1 receptor agonist	9		5	
Sodium-glucose co-transporter 2 inhibitor	10		1	
Sulfonylurea	2		1	
Insulin	6		6	
Angiotensin-converting-enzyme inhibitor	6		4	
Beta blocker	5		5	
Angiotensin II receptor antagonist	2		1	
Calcium channel blocker	1		5	
Thiazide	2		7	
Statins	11		9	

Table 3.2. Cardiovascular and hemodynamic outcomes before and after dietary supplementation of glycocalyx precursors (DSGP) vs. placebo treatment in Veterans with type 2 diabetes. **P*<0.05 vs. Before. $^{\#}P$<0.05, main effect of group. $^{@}P$<0.05 vs. Before DSGP. AUC, area under the curve. CBV, capillary blood volume. MVHS, microvascular health score. PBR, perfused boundary region. FMD, flow-mediated dilation. cfPWV, carotid-femoral pulse wave velocity. MAP, mean arterial pressure. SkM, skeletal muscle. TPR, total peripheral resistance. Measurements were performed at baseline and at 60 minutes of systemic insulin infusion (with the coinfusion of dextrose to maintain euglycemia). The difference between time points was then calculated to capture the insulin response as the outcome variable. Data are presented as means ± SEM.

	Placebo Mean ± SE (n=12)		**DSGP Mean ± SE (n=10)**	
Femoral artery	**Before**	**After**	**Before**	**After**
Baseline diameter (mm)	5.99 ± 0.3	5.95 ± 0.2	6.65 ± 0.2	6.63 ± 0.2
Peak diameter (mm)	6.12 ± 0.3#	6.05 ± 0.2	6.85 ± 0.2	6.85 ± 0.2
Absolute change in diameter (mm)	0.13 ± 0.02#	0.11 ± 0.02	0.20 ± 0.03	0.22 ± 0.04
FMD (%)	2.14 ± 0.27#	1.87 ± 0.34	2.93 ± 0.39	3.20 ± 0.47
Mean shear (s^{-1})	44 ± 8	43 ± 5	52 ± 12	40 ± 7
Shear AUC (a.u.)	10430 ± 2597	9002 ± 1738	12031 ± 4416	7072 ± 1712
Time-to-peak dilation (s)	98 ± 10	107 ± 13	100 ± 11	90 ± 13
Brachial artery				
Baseline diameter (mm)	4.25 ± 0.2	4.24 ± 0.2	4.70 ± 0.2	4.62 ± 0.2
Peak diameter (mm)	4.38 ± 0.2	4.35 ± 0.2	4.84 ± 0.2	4.77 ± 0.2
Absolute change in diameter (mm)	0.13 ± 0.01	0.11 ± 0.02	0.15 ± 0.03	0.15 ± 0.03

FMD (%)	3.20 ± 0.38	2.63 ± 0.54	3.26 ± 0.76	3.40 ± 0.72
Mean shear (s^{-1})	140 ± 20	128 ± 16	119 ± 12	116 ± 11
Shear AUC (a.u.)	25209 ± 2978	19835 ± 2372	22220 ± 1831	19821 ± 2741
Time-to-peak dilation (s)	83 ± 9	77 ± 10	93 ± 8	89 ± 10
GlycoCheck-derived variables				
$CBV_{dynamic}$ (pL/mm^2)/10^3 μm^3	15.5 ± 1.9	15.6 ± 3.0	18.3 ± 2.6	16.5 ± 1.8
$MVHS_{dynamic}$ (µm)	2.8 ± 0.4	3.0 ± 0.6	3.5 ± 0.5	3.1 ± 0.4
Capillary density 4-25µm ($\mu m/mm^2$)	190.2 ± 37.0	170.6 ± 42.1	215.2 ± 39.5	174.7 ± 26.7
$PBR_{4-25\mu m}$ (µm)	2.3 ± 0.2	2.2 ± 0.1	2.1 ± 0.1	2.2 ± 0.1
Additional cardiovascular outcomes				
cfPWV (m/s)	8.45 ± 0.31	8.46 ± 0.44	8.76 ± 0.52	8.70 ± 0.42
24-hour MAP (mmHg)	96 ± 3	96 ± 3	102 ± 3	104 ± 4
Leg blood flow response to insulin (mL/min)	16.93 ± 15.38	9.54 ± 9.03	5.97 ± 11.31	2.97 ± 11.52
Leg vascular conductance response to insulin (a.u.)	0.20 ± 0.18	0.07 ± 0.09	0.01 ± 0.11	0.02 ± 0.11
SkM perfusion response to insulin (A x β value)	-0.17 ± 0.19@	0.04 ± 0.18	1.58 ± 0.68	0.12 ± 0.25*

TPR response to insulin (a.u.)	0.02 ± 0.11	0.26 ± 0.13	0.19 ± 0.29	0.23 ± 0.09

CHAPTER 4: SUMMARY, LIMITATIONS, & FUTURE DIRECTIONS

Summary

This dissertation tested the overarching hypothesis that DSGP would ameliorate vascular dysfunction in Veterans with T2D. The importance of this works stems from the increasing evidence that T2D results in degradation of the endothelial glycocalyx (60, 61, 65, 70, 142, 152, 168–170), and that this phenomenon contributes to endothelial dysfunction, and subsequently, CVD (67). A critical function of the endothelial glycocalyx is to serve as a mechanosensor of shear stress (66), with both *in vitro* and *in vivo* evidence documenting that shear stress provides a crucial mechanical signal to the vascular endothelium that results in the production of NO and other vascular protective molecules (183, 184). Impaired shear stress mechanotransduction is caused, in part, by glycocalyx degradation and is a vital contributor to endothelial dysfunction and CVD complications. As such, the endothelial glycocalyx is a promising vascular therapeutic target for individuals with T2D where glycocalyx degradation manifests. Thus, taken together, this dissertation tested the overarching hypothesis through two specific aims.

Firstly, this dissertation sought to document that DSGP rectifies endothelial glycocalyx integrity in Veterans with T2D. It was hypothesized that Veterans with T2D who receive DSGP will exhibit enhanced glycocalyx integrity compared to Veterans with T2D who receive placebo pills. Subsequently, three approaches were used to test this hypothesis. The first approach hypothesized that endothelial glycocalyx length in en face aortic explants from db/db mice treated with DSGP for four weeks would be increased compared to db/+ control mice and db/db mice treated with vehicle for four weeks. The second approach hypothesized that glycocalyx precursor treatment in human umbilical vein endothelial cells exposed to neuraminidase to induce glycocalyx degradation would result in greater glycocalyx growth compared to vehicle treatment. The third approach hypothesized that DSGP for eight weeks in Veterans with T2D would display improvements in glycocalyx integrity compared to those who received placebo pills. Contrary to the hypothesis, while improvements in glycocalyx length and growth were documented in db/db mice and glycocalyx-degraded human umbilical vein endothelial cells, respectively, DSGP did not enhance glycocalyx integrity relative to placebo in Veterans with T2D.

Secondly, this dissertation attempted to demonstrate the critical role of the endothelial glycocalyx in overall vascular function in Veterans with T2D. The first approach hypothesized that isolated mesenteric arteries and thoracic aortas from db/db mice treated with DSGP for four weeks would improve vascular function relative to db/+ control mice or db/db mice treated with vehicle for four weeks. The second approach hypothesized that DSGP for eight weeks in Veterans with T2D would improve overall vascular function relative to those who received placebo pills. Contrary to the hypothesis, while improvements in isolated mesenteric arteries and thoracic aortas from db/db mice treated with DSGP for four weeks increased vascular function, eight weeks of DSGP did not augment overall vascular function relative to placebo in Veterans with T2D.

Collectively, this dissertation demonstrates that DSGP does not rectify degraded glycocalyx structures in Veterans with T2D. The finding that DSGP did not improve glycocalyx integrity nor overall vascular function in humans is supported by another study in older adults (69), suggesting that DSGP as a therapeutic strategy to enhance vascular function in humans is likely not efficacious. Although speculative, this may be attributable to the fact that several study participants were using SGLT2 inhibitors and GLP-1 receptor agonists, agents with documented beneficial cardiovascular effects (159, 160). This dissertation expands upon previous work by demonstrating that DSGP is beneficial for restoring glycocalyx integrity in a mouse model of T2D (152), but not in humans. Therefore, additional novel ideas to augment glycocalyx structures in T2D and other disease states are warranted. Future studies are needed to determine if specific ingredients of Endocalyx® in isolation, or combined with antidiabetic agents, and/or exercise, has other beneficial effects on T2D and the mechanisms responsible for such improvements.

Limitations

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

Experimental protocol in mice

Lack of male mice

Due to availability, isolated mesenteric and thoracic aortas from female db/db and db/+ mice were used. This mouse model did not match the sex composition of the study participants from the human clinical trial, which were mostly male.

Experimental protocol in humans

Lack of females to investigate potential sex-differences

In this double-blinded, randomized, placebo-controlled clinical trial, there was an unequal distribution of males (n=21) and females (n=1) recruited. Thus, the clinical trial was not statistically powered to observe potential sex differences. This was in part due to approximately 80% of patients at the VA being males and no recruitment strategies were in place to maintain an equal number of male and female participants.

Endothelial glycocalyx integrity assessment and reproducibility

The assessment of glycocalyx integrity in humans was performed sublingually using the non-invasive GlycoCheck^{TM} system. There are no other methods to directly assess glycocalyx integrity in humans. It cannot be attested that an index of glycocalyx integrity in the sublingual circulation represents glycocalyx integrity in other vascular beds. It is plausible that due to the systemic nature of DSGP, an improvement in glycocalyx integrity in one vascular bed should mirror improvements in glycocalyx integrity in all vascular beds, even if the magnitude of change is nonuniform.

Reliable and validated, non-invasively assessed, clinical parameters of the microcirculation and glycocalyx integrity are paramount. A recent publication investigated the reproducibility and validity of the GlycoCheck^{TM} in 49 healthy, young volunteers free of chronic illness or oral disease/injury (188). Intra- and Interobserver variability was measured by having two experienced observers perform three consecutive GlycoCheck^{TM} measurements. When parameters were performed once intraobserver values were poor (intraclass correlation coefficients <0.4). However, when measurements were taken in triplicate and averaged, intraobserver values increased to good (intraclass correlation coefficients >0.6). Additionally, interobserver values for various parameters were documented to be mostly fair. Taken together, the results from this study showed the GlycoCheck^{TM} software has acceptable reliability and reproducibility when consecutive measurements were made and averaged and that repeated measurements are performed ideally via the same observer (188).

Unfortunately, inter- and intraobserver variability of this technique are poor when measuring microcirculatory parameters in pathological conditions (63, 189, 190). However, when averaging multiple consecutive measurements, intraobserver variability classifications were increased to “excellent” in pathological conditions, such as critical illness and smoking (63, 189). Whether this is due to the biological variability that is caused by pathological conditions or due to validity concerns in the GlycoCheck^{TM} software remains unknown. Within this dissertation, collection of microcirculation parameters via the GlycoCheck^{TM} were obtained using consecutive measures, as the software evolved over the years and required more stringent requirements for data capture (178). However, given that the individuals recruited in this dissertation were Veterans with T2D, it is possible that biological variability owing to pathological conditions presented deficiencies in extrapolation for this population. Participants were asked to fast before study visits and refrain from tobacco, diabetic medication, and caffeine intake, however, it cannot be excluded that changes in hydration status, different types of antidiabetic medications, or disease comorbidity status could have impacted the GlycoCheck^{TM} measured outcomes. Future studies are warranted for more standardized conditions before and during data acquisition, as well as determining the reliability and validity of the GlycoCheck^{TM} system in more pathological conditions, such as T2D.

Failure to improve glycocalyx integrity or overall vascular function

Several participants in the DSGP treatment arm of the clinical trial were on antidiabetic medications. Previous studies have shown the beneficial cardiovascular effects of antidiabetic agents, such as SGLT2 inhibitors and GLP-1 receptor agonists (159, 160). However, excluding these antidiabetic drugs would have significantly hindered recruitment in this study population. Additionally, Veterans with T2D were recruited from the local VA hospital and were receiving usual standards of medical care. It is possible that the observed dysfunction in the endothelial glycocalyx and vasculature of study participants may be attributed to the effectiveness of their current medications. Without these treatments, there might have been greater impairments, potentially allowing for more pronounced improvements with the study intervention.

Several individuals in this dissertation were taking insulin as part of their normal standard of care. Insulin therapy is recommended for patients with T2D to achieve glycemic control when other treatments and medications fail to control blood glucose concentrations (191). Evidence suggests that insulin therapy improves vascular function (27, 192, 193), however, given the evidence that decreased insulin sensitivity and reduced beta cell function can simultaneously be present at varying degrees (194), insulin use has been associated with increased risk of mortality, adverse cardiovascular events, and kidney disease (191). Factors such as weight gain, hypoglycemia, and hyperinsulinemia have been shown to be contributors to the negative outcomes experienced with insulin therapy (195, 196). It is likely that the impact of insulin therapy on cardiometabolic outcomes in patients with T2D depends on the severity of insulin resistance present, with high insulin resistance being associated with increased likelihood of mortality, cardiovascular events, and kidney disease prevalence (197).

The group average HOMA-IR value was indicative of severe insulin resistance (3.7mg/dL) (198) and thus, the use of insulin therapy in these highly insulin resistant individuals may have contributed to the lack of effect DSGP was able to produce. Interestingly, individuals who were randomized to receive DSGP treatment also had increases in their HOMA-IR, fasting insulin concentrations, body weight, and percent body fat mass following the eight-week DSGP treatment. Future studies should investigate the impact that insulin therapy has on glycocalyx integrity and overall vascular function in Veterans with T2D taking DSGP as a potential therapeutic to improve vascular health and glycocalyx length.

Absorption rates in T2D are impaired

Previous evidence exists documenting that individuals with T2D have impaired absorption of nutrients (199) and drugs (200). While the exact mechanisms are unknown, there are reports that T2D may affect the absorption and pharmacokinetics due to 1) decreases in gastric emptying and subcutaneous adipose and skeletal muscle blood flow, 2) kidney excretion impairments, and 3) impairments in transportation of drugs and nutrients due to non-enzymatic glycation of albumin (200). Hyperglycemia itself has a significant role in gastrointestinal tract motility, or the movement of food from the mouth through the throat, stomach, small and large intestines, and out of the body. This is due to suppression of antral contraction, increased pyloric contractions, and dysrhythmias in gastric electrical activity (201).

It is well known that in insulin resistant and/or obese individuals, commonly coexisting in T2D, there are ~ 50% reductions in subcutaneous blood flow compared to healthy and non-overweight individuals (202). Additionally, skeletal muscle blood flow at rest and following exercise is compromised in T2D, which can also lead to reduced absorption rates of drugs that use intramuscular administration routes (34, 200, 203–205). With impairments in adipose tissue and skeletal muscle blood flow, along with bacterial overgrowth and altered gut motility (206), this could have limited the distribution of the supplement systemically. Additionally, T2D has shown to decrease absorption rates via dysfunction of the gut microbiota (207, 208). Healthy gut microbiota supports nutrient absorption via breakdown of complex carbohydrates, synthesis of vitamins, produce short-chain fatty acids necessary for appetite and influencing glucose metabolism, and help maintain immune system support for maintenance of gut barrier integrity (207). In T2D, an imbalanced microbiota, known as dysbiosis, impairs absorption rates via 1) reductions in enzyme production, 2) increased intestinal permeability, which may trigger inflammation and independently affect nutrient absorption, 3) increases in bacterial growth, which can compete with the host for essential nutrient utilization, and 4) disruption of gut motility and bile acid metabolism (207–209).

Interestingly, the biguanide metformin, an initial and vital medication to manage hyperglycemia in individuals with T2D, has been shown to impair absorption rates (210). Given that T2D and metformin directly impair absorption rates, it is plausible to speculate that the participants randomly assigned to DSGP treatment within this dissertation had malabsorption of the compounds and thus, were not able to achieve the clinical benefits that the supplement proposes. Indeed, 70% of the participants within the DSGP treatment group in this dissertation were on biguanides, which could have introduced more variability, and thus, be classified as a limitation. Future research studies utilizing DSGP should investigate the extent of the influence common anti-diabetic or obesity medications have on nutrient absorption rates in clinical populations, such as T2D.

The effects of GLP-1 receptor agonists in T2D

Currently, GLP-1 receptor agonists are one of the most effective anti-obesity medications available for treatment in T2D (211). By delaying gastric emptying and gut motility, and by effecting satiety in humans, GLP-1 receptor agonists promote ~ five to seven kilograms of weight loss (211) and that the weight loss may be associated with decreases in visceral and truncal adipose tissue (212, 213). Further, activation of gastric mechano-receptors when the stomach is stretched causes signaling cascades in appetite regulation that are sent to the brain via the vagal nerves (211). With GLP-1 receptor agonists delaying gastric emptying and gut motility, the amount of gastric distention and volume in response to food intake is modulated (214), thus contributing to satiety and aiding in the observed clinically significant loss in body weight. Alongside the known cardiovascular benefits (160), utilization of this anti-obesity agent could have minimized the beneficial impact of DSGP within this dissertation due to the weight loss effects that come with it. With advances in anti-diabetic medications having multiple benefits beyond glucose management (215), and expectations for them to continue to improve, future research studies should be cognizant of the impact of these medications and consider their influence when investigating therapeutic modalities in clinical populations.

Small sample size, randomization, and length of study intervention

In this double-blinded, randomized, placebo-controlled clinical trial, there were a total of 22 participants that successfully completed the intervention. The 1:1 randomization method utilized is effective for measuring group differences in clinical trials with large sample sizes and ensures equal chances for participants to be assigned to either group. However, with the small sample size, this randomization method had the potential to influence the results by chance. A different type of randomization, such as block randomization, may have allowed for better balance between the two groups. Lastly, the study intervention was eight weeks in length, which is a short timeline and can be considered a limitation. The participants recruited in the DSGP group had an average T2D diagnosis length of 15 years and were older adults. Likely, with the age and length of T2D diagnosis, eight weeks could be insufficient in providing clinically relevant improvements in overall vascular function in patients with T2D, especially given the severity of insulin resistance present.

To further highlight the challenges of lifestyle modifications in improving vascular function in T2D, a meta-analysis of vitamin supplementation in humans with T2D was conducted ranging from short- to long-term (four-12 months) (216). Results from this meta-analysis concluded that these variable duration of intervention timelines were incapable of improving vascular function in T2D. Additionally, six months of aerobic exercise has also been shown to be insufficient in improving vascular function in T2D (22). Perhaps longer treatment interventions (>one year) are needed to induce meaningful, clinically relevant improvements in vascular function in humans. It is likely that the age of individuals, length of T2D diagnosis, and the degree of insulin resistance severity play critical roles in therapeutic attempts to attenuate vascular dysfunction in T2D. Future research is warranted to better understand these critical factors and their influence in setting clinical objectives and relevant improvements in vascular function following treatment interventions.

Future directions

Determine if SGLT2 inhibitors and GLP-1 receptor agonists, combined with other therapeutic modalities, are effective in restoring glycocalyx integrity in Veterans with T2D

T2D is widespread among the veteran population and contributes heavily to the growing burden of CVD and cardiovascular mortality in this population (3, 5). As stated in the limitations section, several participants in the DSGP treatment arm of this clinical trial were on SGLT2 inhibitors and GLP-1 receptor agonists. While these medications have beneficial cardiovascular effects, they also have shown improvements in glycocalyx integrity after 12 months of use in T2D (217). While speculative, perhaps these medications would prove efficacious in restoring glycocalyx structures and overall vascular function in Veteran with T2D, who present with their own unique health challenges (218). In line with this, while SGLT2 inhibitors and GLP-1 receptor agonists are effective in improving cardiovascular outcomes, they present risks such as loss of lean body mass and grip strength (219), strong predictors of all-cause and cardiovascular mortality (220). Combining these medications with a resistance exercise training intervention might produce the most favorable results that either independently, or synergistically, improve not only glycocalyx integrity and overall vascular function, but also improve, or at least, maintain lean muscle mass and grip strength. Future studies are needed to test this potential prospect of combining both therapeutic modalities for maximizing glycocalyx and vascular improvements.

Determine the impact of lipotoxicity on endothelial glycocalyx integrity in T2D

Overnutrition and disordered metabolism, commonly found in T2D, results in hyperlipidemia, which is an independent contributor to both endothelial dysfunction and insulin resistance (221). Elevated concentrations of triglycerides, LDL, and free fatty acids (FFA) in the blood stream or in tissues, collectively known as lipotoxicity, damages cardiometabolic health (222–224). The proposed mechanisms by which lipid-mediated toxicities contribute to cardiometabolic dysfunction are increases in oxidative stress, inflammation, mitochondrial dysfunction, endoplasmic reticulum stress, and cell death (221). As mentioned previously, T2D patients are characterized by chronic inflammation (225). During conditions of metabolic stress, cells respond by increasing mitochondrial biogenesis (226) in a compensatory effort for the increased cellular energy demands. Eventually, maladaptation of this process can occur, leading to mitochondrial dysfunction and declines in the production of adenosine triphosphate, the energy necessary for cells to function (221). This dysfunction is associated with decreased fat oxidation and increased intramuscular lipid content (227).

Within this dissertation, measurements of inflammation, mitochondrial function, or FFA concentrations were not assessed. It has been shown that these factors influence not only cardiometabolic health, but also endothelial glycocalyx integrity (228–230). The participants in this dissertation were obese, as defined by body mass index, and had high levels of body fat. While measurements of inflammation, mitochondrial function, or FFA concentrations were not assessed directly, it is appropriate to suspect, based off the body fat and body mass index, that lipotoxicity is a primary contributor to the notable vascular dysfunction observed in this clinical trial. While speculative, the proposed benefits of DSGP could have had a beneficial impact on one of the mechanisms that cause lipotoxicity to exert its deleterious effects on the vascular system. With multiple mechanisms present with lipotoxicity, the potential beneficial effects were not measurable at the level of overall vascular function due to multiple pro-inflammatory and dysfunctional mechanisms masking potentially small improvements. Indeed, evidence suggests that FFA and mitochondrial dysfunction impair PI3K/Akt/eNOS signaling pathways and induce vascular dysfunction (231–233). Inflammation, mitochondrial dysfunction, and FFA have shown to degrade the glycocalyx individually in pre-clinical models (61, 142) while the human literature is more limited. Future research studies should investigate measures of inflammation, mitochondrial function, and FFA concentrations, in combination with the comprehensive vascular phenotyping presented in this dissertation, in humans, to better translate findings from pre-clinical models and detect, systemically, potential improvements in cardiometabolic health parameters in clinical populations, such as T2D.

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233. Qu K, Yan F, Qin X, et al. Mitochondrial dysfunction in vascular endothelial cells and its role in atherosclerosis. *Front Physiol*. 2022;13:1084604.

APPENDIX I – CURRICULUM VITAE

EDUCATIONAL BACKGROUND

Ph.D. Nutrition and Exercise Physiology, 3.90 GPA 2018-Present
University of Missouri, Columbia, MO
Department of Nutrition and Exercise Physiology *Advisors*: Jaume Padilla, PhD & Camila Manrique, MD
Dissertation: "Restoration of the vascular endothelial glycocalyx in veterans with type 2 diabetes."

M.S. Exercise Physiology, 3.58 GPA 2016-2018
West Virginia University School of Medicine, Morgantown, WV
Division of Exercise Physiology *Advisor*: Paul D. Chantler, PhD
Thesis: "The Effects of High Intensity Interval Training on Arterial Health."

B.S. Exercise Science, 3.64 GPA (*Cum Laude*) 2012-2016
Kent State University, Kent, OH
Department of Education, Health, and Human Services

PROFESSIONAL EXPERIENCE

Graduate Research Assistant, *University of Missouri, Columbia, MO* August 2018-Present

- Recruit and coordinate scheduling appointment sessions with research study participants
- Collect and analyze data across multiple clinical trials

Graduate Teaching Assistant, *University of Missouri, Columbia, MO* Fall 2021, Fall 2022

NEP 3850W, Physiology of Exercise

- Lectured and graded 13-14 undergraduate students across basic exercise physiology lab equipment and techniques with final class averages of ~90%
- Organized lectures on how to scientifically write, create graphs, and interpret data
- Collected and analyzed data for weekly email distribution and assignments

Adjunct Instructor, *Clayton State University, Morrow, GA*

HFMG 4060, Personal Nutrition Spring 2019

- Instructor of Record, 100% online course (Brightspace, D2L) of 24 undergraduate students

- Assisted in exercise prescription and dietary analysis of ~25 firefighters (pilot study “Fit for Fire”) in collaboration with HFMG Department and Riverdale Fire Department
- Incorporated dietary analysis from “Fit for Fire” within HFMG 4060, providing students opportunity to analyze firefighter’s dietary habits and offer constructive feedback

INVITED ORAL PRESENTATIONS and GUEST LECTURES

University of Missouri-Columbia

- NEP 3850W Physiology of Exercise: *Temperature Regulation* October 2022
- Mizzou Three Minute Thesis: *10 Days Is Enough* October 2022
- NEP 1034H Intro to Human Nutrition (Honors): *Young Women are Protected Against Vascular Insulin Resistance Following Short-Term Inactivity and Soda Consumption* September 2022

West Virginia University School of Medicine

- EXPH 369 Strength and Conditioning: *Alternative Modes of Training* October 2017

ABSTRACTS and POSTER PRESENTATIONS

1) **Smith J,** Gerhart H, Stavres J, Glickman E, Fennell C, Draper S, Seo Y (2016). Gender differences in running memory and mood state during submaximal exercise in normobaric hypoxia. *Undergraduate Research Symposium,* Kent State University, Kent, OH.
2) Cobb J, Marshall K, **Smith J,** Chantler P, Olfert I (2018). Acute Cardiovascular Effects of Electronic Cigarette Use in Humans. *Van Liere Research Day*, West Virginia University.
3) **Smith J**, Sales A, Park L, Nogueira-Soares R, Woodford M, Padilla J, Manrique-Acevedo C (2020). Young women are protected against leg endothelial dysfunction induced by adoption of a Westernized lifestyle. *Cardiovascular Day Conference,* University of Missouri.
4) Lloyd I, **Smith J**, Ready S, Harper J, Houghton K, Manrique-Acevedo C, Padilla J, Limberg J (2020). Lack of an association between insulin-stimulated peripheral and cerebrovascular vasodilation in healthy young adults. *The Annual Biomedical Research Conference for Minority Students.*
5) Shariffi B, Harper J, **Smith J**, Ready S, Lloyd I, Manrique-Acevedo C, Padilla J, Limberg J (2021). Differential Vasomotor Effects of Insulin in the Peripheral and Cerebrovascular Circulations of Healthy Young Adults. *Experimental Biology*.
6) Lloyd I, Shariffi B, Harper J, **Smith J**, Manrique-Acevedo C, Padilla J, Limberg J (2021). Effect of hyperinsulinemia on cerebral autoregulation. *Undergraduate Research and Creative Achievements Forum,* University of Missouri.

7) Shariffi B, Lloyd I, Wagner J, Harper J, McMillan N, **Smith J**, Manrique-Acevedo C, Padilla J, Limberg J (2022). Effect of Hyperinsulinemia on Cerebral Autoregulation and Myogenic Control of Cerebral Blood Flow in Healthy Young Adults. *Experimental Biology*.
8) **Smith J**, Soares R, Burr K, Ramirez-Perez F, Martinez-Lemus L, Padilla J, Manrique-Acevedo C (2022). Diet-Induced weight loss improves vascular insulin sensitivity in patients with type 2 diabetes. *Missouri Physiological Society Annual Meeting*, University of Missouri.
9) **Smith J**, Soares R, Burr K, Ramirez-Perez F, Martinez-Lemus L, Padilla J, Manrique-Acevedo C (2022). Diet-Induced weight loss improves vascular insulin sensitivity in patients with type 2 diabetes. *CAFNR Research Symposium,* University of Missouri.
10) **Smith J**, Soares R, Burr K, Ramirez-Perez F, Martinez-Lemus L, Padilla J, Manrique-Acevedo C (2023). Diet-Induced weight loss improves vascular insulin sensitivity in patients with type 2 diabetes. *American Physiological Society,* Long Beach, CA.

PEER-REVIEWED PUBLICATIONS

1) Limberg J, **Smith J**, Soares R, Harper J, Houghton K, Jacob D, Mozer M, Grunewald Z, Johnson B, Curry T, Baynard T, Manrique-Acevedo C, Padilla J. Sympathetically mediated increases in cardiac output, not restraint of peripheral vasodilation, contribute to blood pressure maintenance during hyperinsulinemia. *Am J Physiol Heart Circ Physiol* 319(1):H162-H170, 2020.
2) Park L, Parks E, Pettit-Mee R, Woodford M, **Smith J**, Sales A, Martinez-Lemus L, 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* 129(2):283-296, 2020.
3) Limberg J, Soares R, Power G, Harper J, **Smith J**, Shariffi B, Jacob D, Manrique-Acevedo C, Padilla J. Hyperinsulinemia blunts sympathetic vasoconstriction: a possible role of β-adrenergic activation. *Am J Physiol Regul Integr Comp Physiol* 320(6):R771-R779, 2021.
4) Soares R, Ramirez-Perez F, Cabral F, Morales-Quinones M, Foote C, Ghiarone T, Sharma N, Power G, **Smith J**, Rector R, Martinez-Lemus L, Padilla J, Manrique-Acevedo C. SGLT2 inhibition attenuates arterial dysfunction and decreases vascular F-actin content and expression of proteins associated with oxidative stress in aged mice. *Geroscience* 44(3):1657-1675, 2022.
5) **Smith J**, Soares R, McMillan N, Jurrissen T, Martinez-Lemus L, Padilla J, Manrique-Acevedo C. Young women are protected against vascular insulin resistance induced by adoption of an obesogenic lifestyle. *Endocrinology* 163(11):bqac137, 2022.
6) Manrique-Acevedo C, Soares R, **Smith J**, Park L, Burr K, Ramirez-Perez F, McMillan N, Ferreira-Santos L, Sharma N, Olver T, Emter C, Parks E, Limberg J, Martinez-Lemus L, Padilla J. Impact of sex and diet-induced

weight loss on vascular insulin sensitivity in type 2 diabetes. *Am J Physiol Regul Integr Comp Physiol.* 324(3):R293-R304, 2023.

7) Foote C, Ramirez-Perez F, **Smith J**, Ghiarone T, Morales-Quinones M, McMillan N, Augenreich M, Power G, Burr K, Aroor A, Bender S, Manrique-Acevedo C, Padilla J, & Martinez-Lemus L. Neuraminidase inhibition improves endothelial function in diabetic mice. *Am J Physiol Heart Circ Physiol*. 325(6):H1337-H1353, 2023.

UNIVERSITY/COMMUNITY SERVICE AND INVOLVEMENT

***Missouri Physiological Society Membership Committee,* Chair** November 2022-Present

- Recruit new members, identify strategies for membership retention, convey membership benefits

Graduate Student Association, **Treasurer** January-May 2019

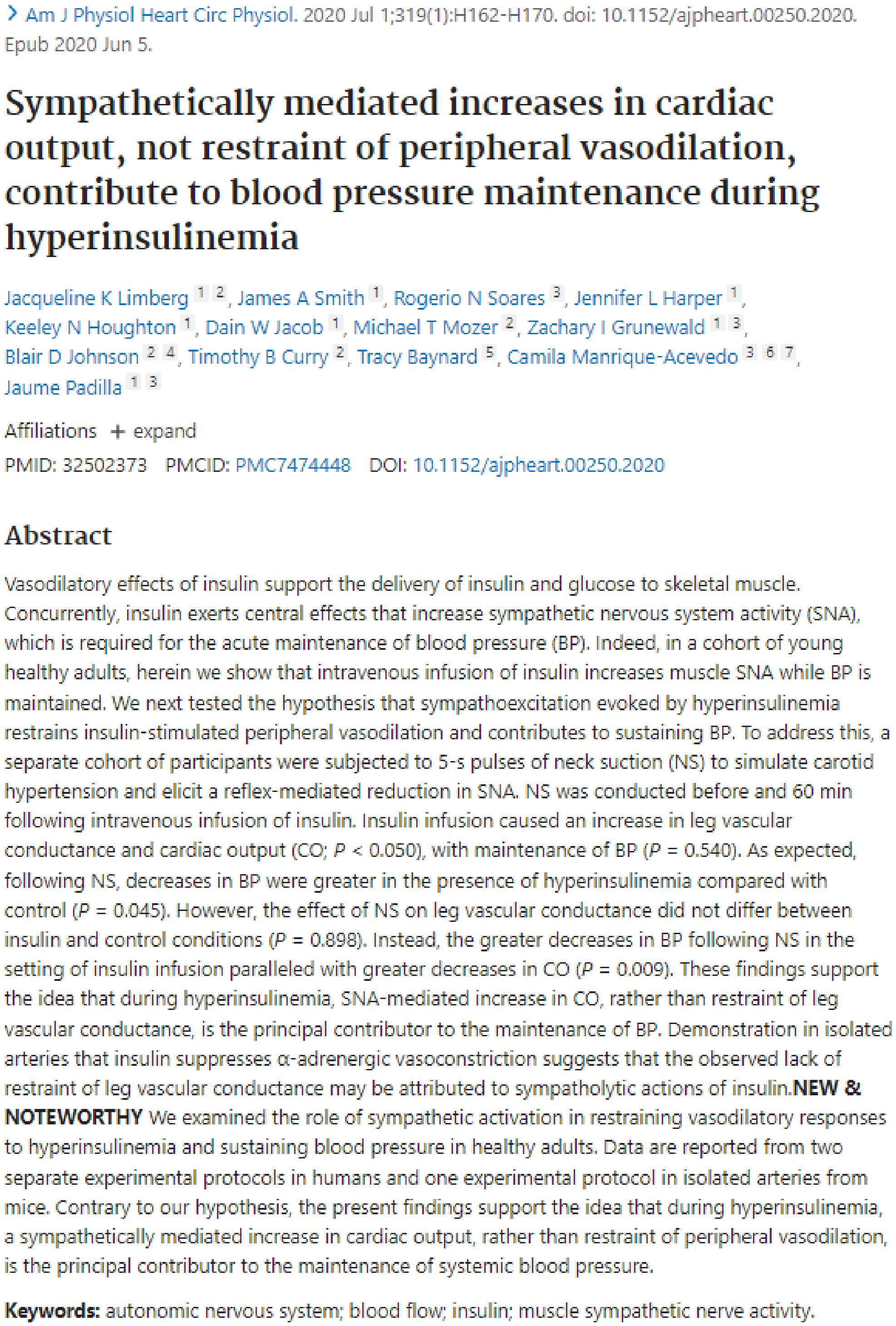
- Assist with funding applications for travel and conferences

Show Me Mizzou, **Volunteer** April 2019

- Ultrasound demonstration for science interested high school students

APPENDIX II – ABSTRACTS OF PEER-REVIEWED PUBLICATIONS

(7)



› Am J Physiol Heart Circ Physiol. 2020 Jul 1;319(1):H162-H170. doi: 10.1152/ajpheart.00250.2020.
Epub 2020 Jun 5.

Sympathetically mediated increases in cardiac output, not restraint of peripheral vasodilation, contribute to blood pressure maintenance during hyperinsulinemia

Jacqueline K Limberg [1, 2], James A Smith [1], Rogerio N Soares [3], Jennifer L Harper [1], Keeley N Houghton [1], Dain W Jacob [1], Michael T Mozer [2], Zachary I Grunewald [1, 3], Blair D Johnson [2, 4], Timothy B Curry [2], Tracy Baynard [5], Camila Manrique-Acevedo [3, 6, 7], Jaume Padilla [1, 3]

Affiliations + expand

PMID: 32502373 PMCID: PMC7474448 DOI: 10.1152/ajpheart.00250.2020

Abstract

Vasodilatory effects of insulin support the delivery of insulin and glucose to skeletal muscle. Concurrently, insulin exerts central effects that increase sympathetic nervous system activity (SNA), which is required for the acute maintenance of blood pressure (BP). Indeed, in a cohort of young healthy adults, herein we show that intravenous infusion of insulin increases muscle SNA while BP is maintained. We next tested the hypothesis that sympathoexcitation evoked by hyperinsulinemia restrains insulin-stimulated peripheral vasodilation and contributes to sustaining BP. To address this, a separate cohort of participants were subjected to 5-s pulses of neck suction (NS) to simulate carotid hypertension and elicit a reflex-mediated reduction in SNA. NS was conducted before and 60 min following intravenous infusion of insulin. Insulin infusion caused an increase in leg vascular conductance and cardiac output (CO; $P < 0.050$), with maintenance of BP ($P = 0.540$). As expected, following NS, decreases in BP were greater in the presence of hyperinsulinemia compared with control ($P = 0.045$). However, the effect of NS on leg vascular conductance did not differ between insulin and control conditions ($P = 0.898$). Instead, the greater decreases in BP following NS in the setting of insulin infusion paralleled with greater decreases in CO ($P = 0.009$). These findings support the idea that during hyperinsulinemia, SNA-mediated increase in CO, rather than restraint of leg vascular conductance, is the principal contributor to the maintenance of BP. Demonstration in isolated arteries that insulin suppresses α-adrenergic vasoconstriction suggests that the observed lack of restraint of leg vascular conductance may be attributed to sympatholytic actions of insulin.**NEW & NOTEWORTHY** We examined the role of sympathetic activation in restraining vasodilatory responses to hyperinsulinemia and sustaining blood pressure in healthy adults. Data are reported from two separate experimental protocols in humans and one experimental protocol in isolated arteries from mice. Contrary to our hypothesis, the present findings support the idea that during hyperinsulinemia, a sympathetically mediated increase in cardiac output, rather than restraint of peripheral vasodilation, is the principal contributor to the maintenance of systemic blood pressure.

Keywords: autonomic nervous system; blood flow; insulin; muscle sympathetic nerve activity.

> 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]

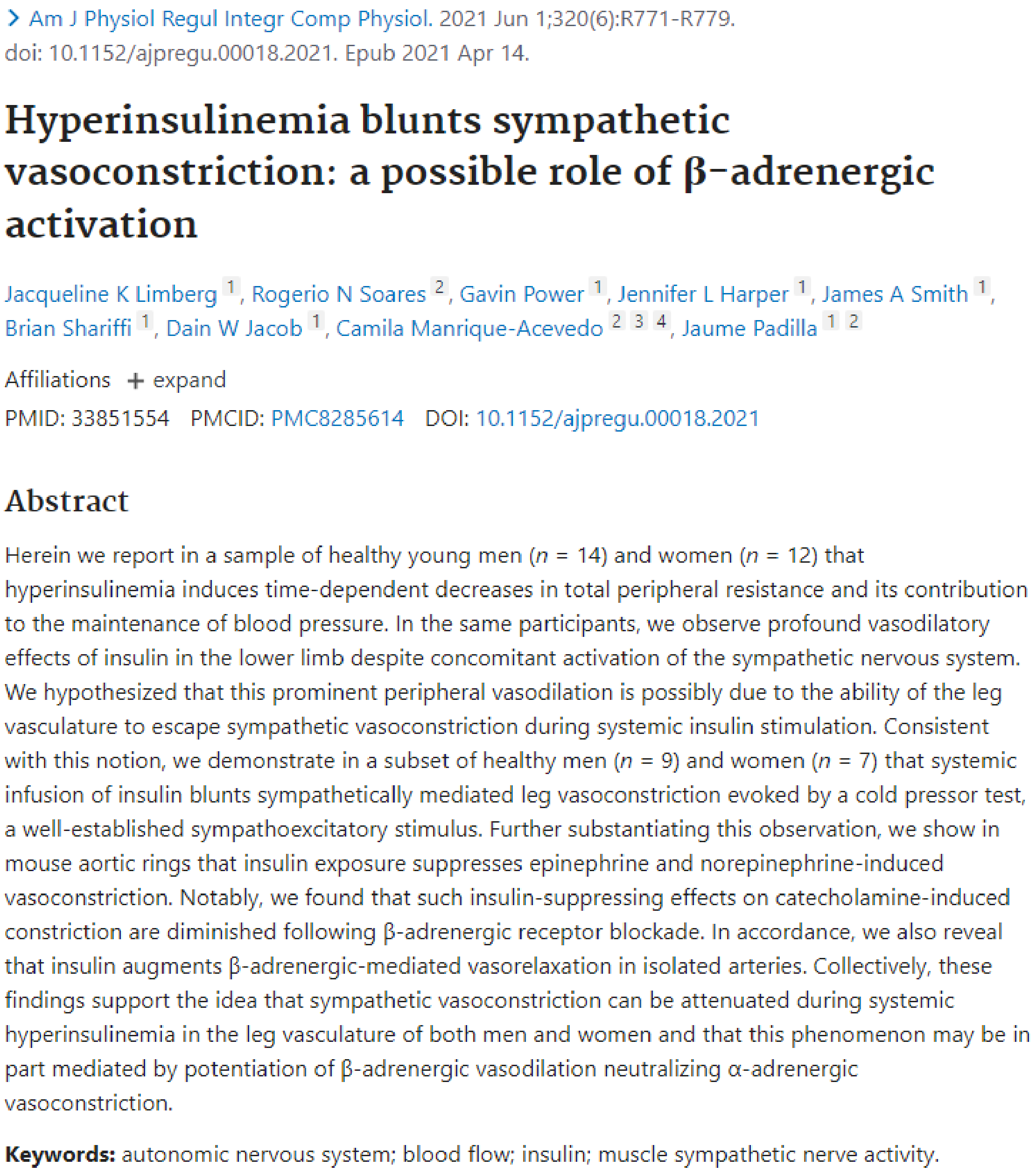
Affiliations + expand

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

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.



› Am J Physiol Regul Integr Comp Physiol. 2021 Jun 1;320(6):R771-R779.
doi: 10.1152/ajpregu.00018.2021. Epub 2021 Apr 14.

Hyperinsulinemia blunts sympathetic vasoconstriction: a possible role of β-adrenergic activation

Jacqueline K Limberg [1], Rogerio N Soares [2], Gavin Power [1], Jennifer L Harper [1], James A Smith [1], Brian Shariffi [1], Dain W Jacob [1], Camila Manrique-Acevedo [2] [3] [4], Jaume Padilla [1] [2]

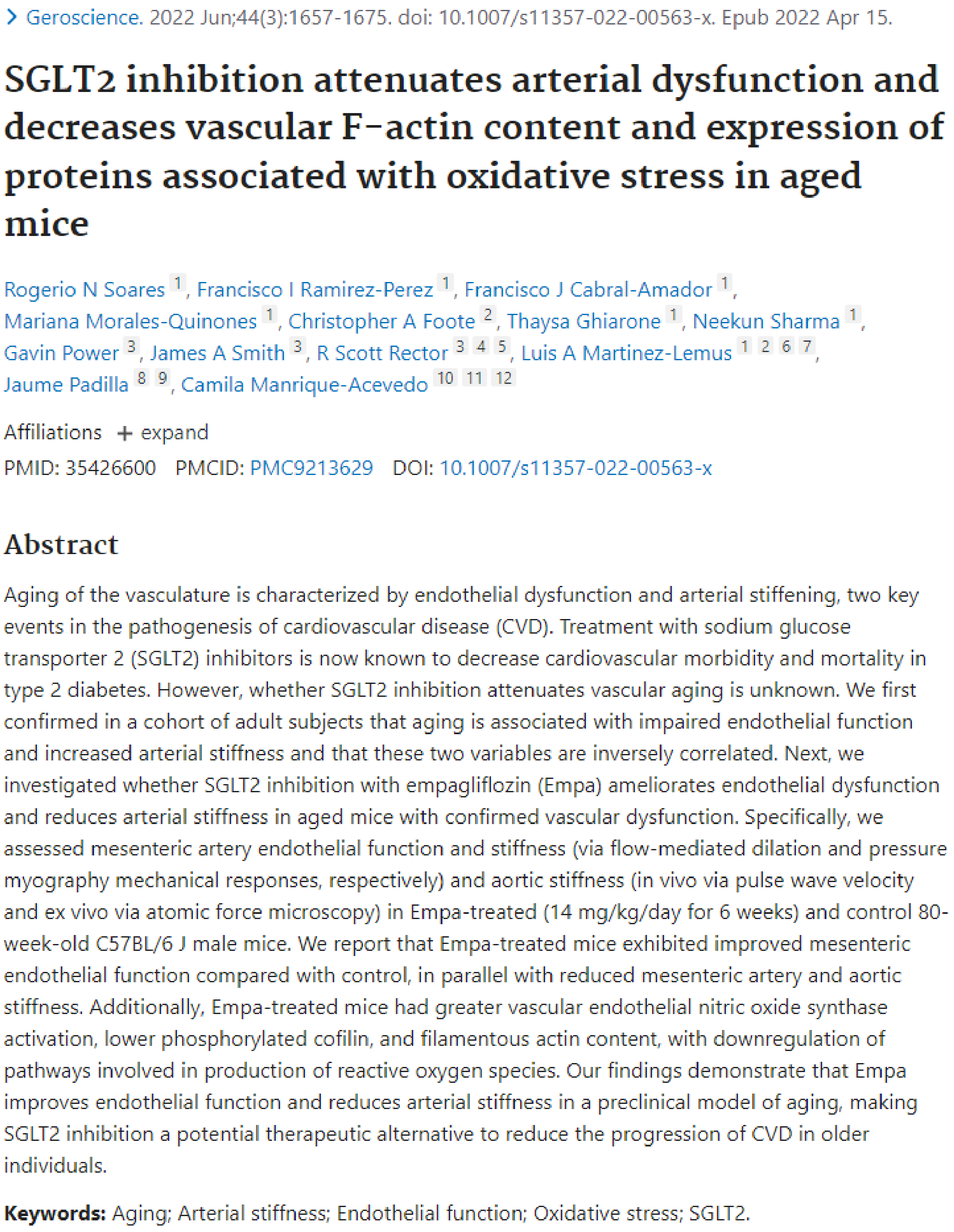
Affiliations + expand

PMID: 33851554 PMCID: PMC8285614 DOI: 10.1152/ajpregu.00018.2021

Abstract

Herein we report in a sample of healthy young men (*n* = 14) and women (*n* = 12) that hyperinsulinemia induces time-dependent decreases in total peripheral resistance and its contribution to the maintenance of blood pressure. In the same participants, we observe profound vasodilatory effects of insulin in the lower limb despite concomitant activation of the sympathetic nervous system. We hypothesized that this prominent peripheral vasodilation is possibly due to the ability of the leg vasculature to escape sympathetic vasoconstriction during systemic insulin stimulation. Consistent with this notion, we demonstrate in a subset of healthy men (*n* = 9) and women (*n* = 7) that systemic infusion of insulin blunts sympathetically mediated leg vasoconstriction evoked by a cold pressor test, a well-established sympathoexcitatory stimulus. Further substantiating this observation, we show in mouse aortic rings that insulin exposure suppresses epinephrine and norepinephrine-induced vasoconstriction. Notably, we found that such insulin-suppressing effects on catecholamine-induced constriction are diminished following β-adrenergic receptor blockade. In accordance, we also reveal that insulin augments β-adrenergic-mediated vasorelaxation in isolated arteries. Collectively, these findings support the idea that sympathetic vasoconstriction can be attenuated during systemic hyperinsulinemia in the leg vasculature of both men and women and that this phenomenon may be in part mediated by potentiation of β-adrenergic vasodilation neutralizing α-adrenergic vasoconstriction.

Keywords: autonomic nervous system; blood flow; insulin; muscle sympathetic nerve activity.



› Geroscience. 2022 Jun;44(3):1657-1675. doi: 10.1007/s11357-022-00563-x. Epub 2022 Apr 15.

SGLT2 inhibition attenuates arterial dysfunction and decreases vascular F-actin content and expression of proteins associated with oxidative stress in aged mice

Rogerio N Soares [1], Francisco I Ramirez-Perez [1], Francisco J Cabral-Amador [1], Mariana Morales-Quinones [1], Christopher A Foote [2], Thaysa Ghiarone [1], Neekun Sharma [1], Gavin Power [3], James A Smith [3], R Scott Rector [3] [4] [5], Luis A Martinez-Lemus [1] [2] [6] [7], Jaume Padilla [8] [9], Camila Manrique-Acevedo [10] [11] [12]

Affiliations + expand

PMID: 35426600 PMCID: PMC9213629 DOI: 10.1007/s11357-022-00563-x

Abstract

Aging of the vasculature is characterized by endothelial dysfunction and arterial stiffening, two key events in the pathogenesis of cardiovascular disease (CVD). Treatment with sodium glucose transporter 2 (SGLT2) inhibitors is now known to decrease cardiovascular morbidity and mortality in type 2 diabetes. However, whether SGLT2 inhibition attenuates vascular aging is unknown. We first confirmed in a cohort of adult subjects that aging is associated with impaired endothelial function and increased arterial stiffness and that these two variables are inversely correlated. Next, we investigated whether SGLT2 inhibition with empagliflozin (Empa) ameliorates endothelial dysfunction and reduces arterial stiffness in aged mice with confirmed vascular dysfunction. Specifically, we assessed mesenteric artery endothelial function and stiffness (via flow-mediated dilation and pressure myography mechanical responses, respectively) and aortic stiffness (in vivo via pulse wave velocity and ex vivo via atomic force microscopy) in Empa-treated (14 mg/kg/day for 6 weeks) and control 80-week-old C57BL/6 J male mice. We report that Empa-treated mice exhibited improved mesenteric endothelial function compared with control, in parallel with reduced mesenteric artery and aortic stiffness. Additionally, Empa-treated mice had greater vascular endothelial nitric oxide synthase activation, lower phosphorylated cofilin, and filamentous actin content, with downregulation of pathways involved in production of reactive oxygen species. Our findings demonstrate that Empa improves endothelial function and reduces arterial stiffness in a preclinical model of aging, making SGLT2 inhibition a potential therapeutic alternative to reduce the progression of CVD in older individuals.

Keywords: Aging; Arterial stiffness; Endothelial function; Oxidative stress; SGLT2.

› Endocrinology. 2022 Oct 11;163(11):bqac137. doi: 10.1210/endocr/bqac137.

Young Women Are Protected Against Vascular Insulin Resistance Induced by Adoption of an Obesogenic Lifestyle

James A Smith [1], Rogerio N Soares [2], Neil J McMillan [1], Thomas J Jurrissen [1],
Luis A Martinez-Lemus [2] [3] [4], Jaume Padilla [1] [3] [5], Camila Manrique-Acevedo [3] [5] [6]

Affiliations + expand

PMID: 35974454 PMCID: PMC10233280 DOI: 10.1210/endocr/bqac137

Erratum in

Correction to: "Young Women are Protected Against Vascular Insulin Resistance Induced by Adoption of an Obesogenic Lifestyle".
[No authors listed]
Endocrinology. 2023 Feb 11;164(4):bqad037. doi: 10.1210/endocr/bqad037.
PMID: 36869675 Free PMC article. No abstract available.

Abstract

Vascular insulin resistance is a feature of obesity and type 2 diabetes that contributes to the genesis of vascular disease and glycemic dysregulation. Data from preclinical models indicate that vascular insulin resistance is an early event in the disease course, preceding the development of insulin resistance in metabolically active tissues. Whether this is translatable to humans requires further investigation. To this end, we examined if vascular insulin resistance develops when young healthy individuals (n = 18 men, n = 18 women) transition to an obesogenic lifestyle that would ultimately cause whole-body insulin resistance. Specifically, we hypothesized that short-term (10 days) exposure to reduced ambulatory activity (from >10 000 to <5000 steps/day) and increased consumption of sugar-sweetened beverages (6 cans/day) would be sufficient to prompt vascular insulin resistance. Furthermore, given that incidence of insulin resistance and cardiovascular disease is lower in premenopausal women than in men, we postulated that young females would be protected against vascular insulin resistance. Consistent with this hypothesis, we report that after reduced ambulation and increased ingestion of carbonated beverages high in sugar, young healthy men, but not women, exhibited a blunted leg blood flow response to insulin and suppressed skeletal muscle microvascular perfusion. These findings were associated with a decrease in plasma adropin and nitrite concentrations. This is the first evidence in humans that vascular insulin resistance can be provoked by short-term adverse lifestyle changes. It is also the first documentation of a sexual dimorphism in the development of vascular insulin resistance in association with changes in adropin levels.

Keywords: adropin; blood flow; endothelial dysfunction; sedentary behavior; sex differences; sugar-sweetened beverages.

Am J Physiol Regul Integr Comp Physiol. 2023 Mar 1;324(3):R293-R304.
doi: 10.1152/ajpregu.00249.2022. Epub 2023 Jan 9.

Impact of sex and diet-induced weight loss on vascular insulin sensitivity in type 2 diabetes

Camila Manrique-Acevedo [1,2,3], Rogerio N Soares [2], James A Smith [2,4], Lauren K Park [4,5], Katherine Burr [2], Francisco I Ramirez-Perez [2], Neil J McMillan [2,4], Larissa Ferreira-Santos [2], Neekun Sharma [2,6], T Dylan Olver [7,8], Craig A Emter [2,7], Elizabeth J Parks [2,4,9], Jacqueline K Limberg [4], Luis A Martinez-Lemus [2,6,10], Jaume Padilla [2,3,4]

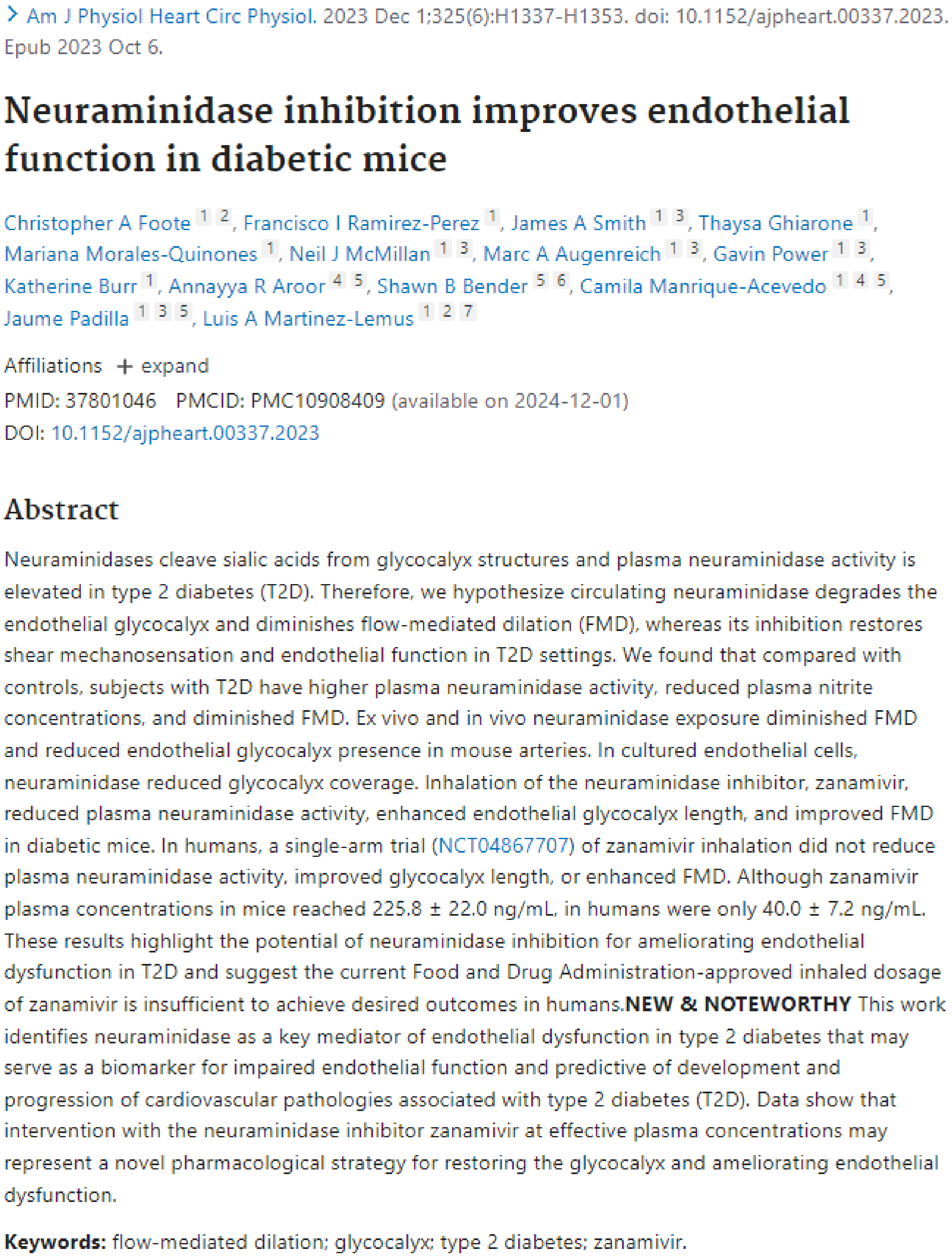
Affiliations + expand

PMID: 36622084 PMCID: PMC9942885 DOI: 10.1152/ajpregu.00249.2022

Abstract

Vascular insulin resistance, a major characteristic of obesity and type 2 diabetes (T2D), manifests with blunting of insulin-induced vasodilation. Although there is evidence that females are more whole body insulin sensitive than males in the healthy state, whether sex differences exist in vascular insulin sensitivity is unclear. Also uncertain is whether weight loss can reestablish vascular insulin sensitivity in T2D. The purpose of this investigation was to *1*) establish if sex differences in vasodilatory responses to insulin exist in absence of disease, *2*) determine whether female sex affords protection against the development of vascular insulin resistance with long-term overnutrition and obesity, and *3*) examine if diet-induced weight loss can restore vascular insulin sensitivity in men and women with T2D. First, we show in healthy mice and humans that sex does not influence insulin-induced femoral artery dilation and insulin-stimulated leg blood flow, respectively. Second, we provide evidence that female mice are protected against impairments in insulin-induced dilation caused by overnutrition-induced obesity. Third, we show that men and women exhibit comparable levels of vascular insulin resistance when T2D develops but that diet-induced weight loss is effective at improving insulin-stimulated leg blood flow, particularly in women. Finally, we provide indirect evidence that these beneficial effects of weight loss may be mediated by a reduction in endothelin-1. In aggregate, the present data indicate that female sex confers protection against obesity-induced vascular insulin resistance and provide supportive evidence that, in women with T2D, vascular insulin resistance can be remediated with diet-induced weight loss.

Keywords: obesity; sex differences; type 2 diabetes; vascular function; vascular insulin resistance.



› Am J Physiol Heart Circ Physiol. 2023 Dec 1;325(6):H1337-H1353. doi: 10.1152/ajpheart.00337.2023. Epub 2023 Oct 6.

Neuraminidase inhibition improves endothelial function in diabetic mice

Christopher A Foote [1] [2], Francisco I Ramirez-Perez [1], James A Smith [1] [3], Thaysa Ghiarone [1], Mariana Morales-Quinones [1], Neil J McMillan [1] [3], Marc A Augenreich [1] [3], Gavin Power [1] [3], Katherine Burr [1], Annayya R Aroor [4] [5], Shawn B Bender [5] [6], Camila Manrique-Acevedo [1] [4] [5], Jaume Padilla [1] [3] [5], Luis A Martinez-Lemus [1] [2] [7]

Affiliations + expand

PMID: 37801046 PMCID: PMC10908409 (available on 2024-12-01)
DOI: 10.1152/ajpheart.00337.2023

Abstract

Neuraminidases cleave sialic acids from glycocalyx structures and plasma neuraminidase activity is elevated in type 2 diabetes (T2D). Therefore, we hypothesize circulating neuraminidase degrades the endothelial glycocalyx and diminishes flow-mediated dilation (FMD), whereas its inhibition restores shear mechanosensation and endothelial function in T2D settings. We found that compared with controls, subjects with T2D have higher plasma neuraminidase activity, reduced plasma nitrite concentrations, and diminished FMD. Ex vivo and in vivo neuraminidase exposure diminished FMD and reduced endothelial glycocalyx presence in mouse arteries. In cultured endothelial cells, neuraminidase reduced glycocalyx coverage. Inhalation of the neuraminidase inhibitor, zanamivir, reduced plasma neuraminidase activity, enhanced endothelial glycocalyx length, and improved FMD in diabetic mice. In humans, a single-arm trial (NCT04867707) of zanamivir inhalation did not reduce plasma neuraminidase activity, improved glycocalyx length, or enhanced FMD. Although zanamivir plasma concentrations in mice reached 225.8 ± 22.0 ng/mL, in humans were only 40.0 ± 7.2 ng/mL. These results highlight the potential of neuraminidase inhibition for ameliorating endothelial dysfunction in T2D and suggest the current Food and Drug Administration-approved inhaled dosage of zanamivir is insufficient to achieve desired outcomes in humans.**NEW & NOTEWORTHY** This work identifies neuraminidase as a key mediator of endothelial dysfunction in type 2 diabetes that may serve as a biomarker for impaired endothelial function and predictive of development and progression of cardiovascular pathologies associated with type 2 diabetes (T2D). Data show that intervention with the neuraminidase inhibitor zanamivir at effective plasma concentrations may represent a novel pharmacological strategy for restoring the glycocalyx and ameliorating endothelial dysfunction.

Keywords: flow-mediated dilation; glycocalyx; type 2 diabetes; zanamivir.

VITA

James Alexander Smith was born in Columbus, Indiana. Following completion of high school in 2012, James attended Kent State University in Kent, Ohio where he received a Bachelor of Science in Exercise Science with a minor in Business in 2016. During his undergraduate studies, James became interested in researching cardiovascular adaptations following exercise. This led him to pursue a Master of Science degree in Exercise Physiology from West Virginia University School of Medicine in Morgantown, West Virginia where he graduated in 2018. Subsequently, James moved to Columbia, Missouri in 2018 to pursue a PhD in Nutrition and Exercise Physiology from the University of Missouri-Columbia under the combined mentorship of Drs. Jaume Padilla and Camila Manrique-Acevedo where he obtained his PhD in 2024.