Novel Mechanisms of Peripheral Vascular Dysfunction in Patients with Type 2 Diabetes

by

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Abstract

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Currently, over 30 million adults in the United States have been diagnosed with Type 2 Diabetes mellitus (T2D). Importantly, T2D disproportionately augments the risk for the development of cardiovascular disease (CVD), which is currently the leading cause of morbidity and mortality in the United States. A hallmark characteristic of T2D is insulin resistance (IR), defined as a reduced tissue responsiveness to insulin stimulation, which contributes to prolonged periods of post-prandial hyperglycemia and is associated with a compensatory rise in pancreatic insulin secretion (hyperinsulinemia). Importantly, there is a well-known bi-directional association between the degree of IR/hyperinsulinemia and hypertension (HTN), with nearly 60% of all patients with T2D progressing to develop HTN. Yet, while this co-existence is well defined, the unifying mechanisms remain incompletely understood. A key pathogenic feature of both IR and HTN is peripheral vascular dysfunction, or dysregulated vascular endothelial cell signaling, typically attributable to greater vasoconstrictor and lesser vasodilator signaling. Beyond its metabolic actions, insulin exhibits both indirect (central nervous system) and direct (local vascular) actions that contribute to the regulation of peripheral vascular tone. In the central nervous system, insulin stimulates an increase in sympathetic nerve activity (SNA) which acts on vascular smooth muscle to confer vasoconstriction. Yet, while patients with T2D exhibit chronic hyperinsulinemia, and thus would be expected to exhibit marked sympatho-excitation, direct recordings of SNA have provided equivocal results. However, quantification of central sympathetic outflow alone represents only one aspect of sympathetic regulation, and much less is known regarding the ensuing vascular smooth muscle contractile response(s) following spontaneous bursts of MSNA (i.e., sympathetic transduction) in T2D. At the local vascular level, insulin signaling also directly stimulates an increase in skeletal muscle blood flow (vasodilation). Insulin-stimulated vasodilation is achieved through insulin binding its target receptor on endothelial cells, and the initiation of two distinct signaling cascades. The first pathway produces nitric oxide (vasodilator), while the second pathway produces endothelin-1 (ET-1; potent vasoconstrictor). Importantly, the skeletal muscle blood flow response to insulin is markedly reduced in patients with T2D, and preclinical animal models of IR/T2D suggest that a shift

towards insulin-induced overproduction of ET-1 may be one putative mechanism. Accordingly, this dissertation has focused on further understanding how sympathetic, and ET-1 mediated vasoconstriction may contribute to T2D-associated vascular dysfunction in humans. First, using a novel spike-triggered averaging methodology developed and validated in the Fadel Laboratory, we quantified sympathetic transduction in patients with T2D (Chapter 2). Then, following the demonstration of an augmented sympathetic transduction in patients with T2D, we further set-out to understand the role that hyperinsulinemia may play in mediating this augmented sympathetic transduction by investigating the effect of acute hyperinsulinemia on sympathetic transduction in man (Chapter 3). Finally, we mechanistically probe the role that ET-1 plays in limiting insulin-stimulated vasodilation via blockade of the ET-1 receptors during hyperinsulinemia in patients with T2D (Chapter 4). In sum, the work contained herein supports role(s) for both greater sympathetic (via way of greater sympathetic transduction), and ET-1 mediated vasoconstriction in T2D-associated vascular dysfunction.

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Chapter 1

Introduction

Type 2 Diabetes (T2D) is a metabolic disease characterized by significant dysregulation of glucose metabolism. Unlike Type 1 Diabetes, which is primarily characterized as an autoimmune disorder, the pathogenesis of T2D is complex, multi-factorial, and perceptually idiopathic (1, 2). Therefore, the diagnosis of T2D currently relies on the manifestation of one of the four following signs of glucose dysregulation: 1) Fasting plasma glucose level ≥ 126 mg/dL; 2) plasma glucose ≥ 200 mg/dL at 2-hours post-oral glucose tolerance test; 3) presentation of acute hyperglycemic symptoms with plasma glucose ≥ 200 mg/dL; or 4) glycated hemoglobin (HbA1C) ≥ 6.5% (1, 2).

Based upon *National Health and Nutrition Examination Survey* (NHANES) data from 2019, it is estimated that ~30 million adults in the United States have been diagnosed with T2D, 9.4 million live with undiagnosed T2D, and an astonishing 91.8 million people exhibit pre-diabetes. Therefore, the age-adjusted prevalence of T2D in United States adults stands at present around 9.4%. Economically, in the United States the total estimated cost of diabetes in 2017 was $327 billion, wherein the cost of treating patients with diabetes amounted to 1 out of every 4 healthcare dollars spent, and more than half of that cost was directly attributable to/associated with diabetic complications (3). After-adjusting for inflation, these numbers represent a 26% increase in the economic cost of care for diabetes in just five years, with this rise in cost due primarily to the increased prevalence of T2D and the increased cost of care per patient associated with advanced treatment (3). Collectively, these data demonstrate that T2D is already a major economic burden in our country. Although alarmingly, projections suggest the prevalence of T2D in the United States will continue to rise to nearly one-third of the population by year 2050 (4). Therefore, in full view of this impending economic and physical burden that will accompany the inevitable country-wide epidemic of T2D, the identification of novel therapeutic targets to reduce the financial burden of T2D should be viewed as a public-health imperative.

A major source of cost in T2D treatment is the accompanying cardiovascular disease (CVD). Indeed, CVD is currently the sole leading cause of morbidity and mortality in the United States, and T2D disproportionately augments the risk for the development of CVD (5, 6). In fact, ~32% of patients with T2D will progress to develop CVD (7), and at present 27-35% of the cost associated with T2D treatment can be attributed specifically to the presence/complication(s) of diabetic-CVD (8). In addition, beyond these direct relationships to CVD, T2D is also highly co-morbid with hypertension (HTN), one of the strongest predictors of CVD-development (9). Indeed, approximately 60% of patients with T2D exhibit HTN in observational/cross-sectional studies (10). Importantly, the magnitude of hyperglycemia is linearly related to CVD risk (11), and despite recent advancements for the treatment of T2D, poor glycemic control persists in nearly half of all patients with T2D (12). Taken together then, poor glycemic control may be viewed as a source of CVD risk which despite all current therapeutic practices has not been effectively abrogated.

One potential contributor to continued glucose intolerance in the T2D population is peripheral vascular dysfunction. Broadly, vascular dysfunction is typically attributed to dysregulated vascular endothelial cell signaling (i.e., endothelial dysfunction), and characterized by enhanced endothelial vasoconstrictor and/or reduced vasodilator signaling. However, in vivo assessments of vascular function inherently include vascular smooth muscle vasoconstriction conferred by the sympathetic nervous system. Despite treatment, vascular dysfunction persists in nearly all cohorts of studied patients with T2D (13-15). Moreover, vascular dysfunction is also associated with the development of HTN (15-17), which may implicate vascular dysfunction as a unifying link between these phenomena (18). Yet, the specific mechanisms of vascular dysfunction in T2D remain to be entirely elucidated.

In T2D, insulin may be a primary mediator of vascular dysfunction. Beyond its metabolic actions, insulin exhibits both indirect (central nervous system) and direct (local vascular) actions that contribute to the regulation of peripheral vascular tone. In health, during the post-prandial state these mechanisms work in tandem to fine tune the redistribution of blood flow away from metabolically inert tissue, toward metabolically-active tissue (e.g., skeletal muscle). However, a hallmark characteristic of T2D is insulin resistance (IR), defined as a reduced tissue responsiveness to insulin stimulation. The net effect of IR is an inability to clear plasma glucose, resulting in prolonged periods of post-prandial hyperglycemia. Acutely, hyperglycemia stimulates greater pancreatic insulin release, thereby compensating for the reduced tissue responsiveness by augmenting insulin secretion. However, chronically, and as IR worsens (e.g., as occurs in T2D), hyperinsulinemia alone is insufficient to maintain normal plasma glucose, resulting in a pathological feed-forward state of chronic hyperglycemic-hyperinsulinemia (19). Therefore, the IR-state, as well as the associated chronic hyperinsulinemia, might plausibly contribute to T2D-induced vascular dysfunction due to persistent central and peripheral insulin signaling.

Within the central nervous system, insulin stimulates sympathetic outflow, which confers vascular smooth muscle vasoconstriction. The expected effect of chronic hyperinsulinemia in T2D would thus be marked sympatho-excitation, favoring excessive sympathetic vasoconstriction. Interestingly though, direct recordings of muscle sympathetic nerve activity (MSNA) in patients with T2D have yielded equivocal results, where some report greater (20, 21), while others report no difference (22, 23) in resting MSNA in patients with T2D. Yet, quantification of MSNA alone does not provide information regarding the ensuing vascular smooth muscle contractile response, and ultimately the blood pressure (BP) responses following spontaneous bursts of MSNA (i.e., sympathetic transduction) in patients with T2D. Therefore, whether or not an exaggerated

sympathetic transduction may contribute to vascular dysfunction in T2D remains unknown. Moreover, the putative effect of augmented sympathetic transduction in T2D on subsequent excursions in BP, and its potential role as a pathogenic link between T2D and HTN remains to be defined.

At the local vascular level, insulin stimulates two distinct signaling pathways, one which produces vasodilation (nitric oxide; NO pathway) and another which stimulates either potent vasoconstriction or vasodilation (endothelin-1; ET-1 pathway). These reciprocal actions of the ET-1 pathway are mediated by distinct receptor sub-populations. Indeed, ET-1 A (ETA) receptors and ET-1 B (ETB) receptors located on vascular smooth muscle both confer vasoconstriction. However, ETB receptors located on endothelial cells result in NO production, and vasodilation. The net effect of these contrasting pathways during hyperinsulinemia in healthy individuals is a substantial increase in skeletal muscle blood flow (i.e., vasodilation), the primary site of glucose disposal (24). These local vascular actions of insulin contribute substantially (~35%) to overall glycemic control (25). Contrastingly though, insulin-stimulated blood flow is markedly impaired in patients with T2D (26), an effect that has been proposed to be mediated by T2D-induced vascular dysfunction associated with local vascular insulin signaling (27, 28). In this regard, preclinical animal models of IR (29), as well as data from skeletal muscle biopsies taken during hyperinsulinemia in patients with T2D (26) generally suggest a shift towards a greater insulin-stimulated production of ET-1. However, whether or not ET-1 mediated vasoconstriction plays a functional role in limiting insulin-stimulated blood flow in patients with T2D, and the distinct contributions of the ETA versus ETB receptor remain to be entirely elucidated.

In summary, while exaggerated sympathetically-mediated vasoconstriction may contribute to T2D-induced vascular dysfunction, and augmented ET-1 mediated vasoconstriction may be a

functional limiter of insulin-stimulated blood flow in T2D, these mechanisms remain decidedly understudied in humans with T2D. Indeed, the majority of information currently available stems from preclinical animal model data, and translation of these findings to humans with T2D is scarce. Accordingly, this dissertation has focused on further understanding how the central and local vascular actions of insulin may contribute to T2D-associated vascular dysfunction in humans. In addition, given the incidence of HTN in this population, particular emphasis has been placed on the implications of the experimental findings for blood pressure (BP) regulation when applicable.

In Chapter 2, we will provide general information regarding the fundamental approaches available for studying vascular function in human health and disease. We also provide a brief historical account of the methodological approaches for assessing sympathetic transduction in humans, and the putative advantages and shortcomings of these techniques. These sections are designed specifically to convey the distinct methodological advantages to the techniques utilized within this dissertation. Specifically, signal-averaging quantifications of sympathetic transduction (as occur in Chapters 3 and 4), as well as the use of intra-arterial infusions to interrogate ET-1 mediated vasoconstriction during hyperinsulinemia (as occurs in Chapter 5). Following these methodological inquiries, we provide an in-depth review of the general features of insulin receptor signal transduction, as well as the central sympatho-excitatory and local vascular actions of insulin, and their interactions in health. We conclude Chapter 2 with the putative effect of IR and T2D on these signaling pathways, culminating in the critical knowledge gaps and experimental hypotheses developed to address these gaps in subsequent Chapters.

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Chapter 2

Literature Review and Experimental Hypotheses

In order to define the mechanisms of peripheral vascular dysfunction in type 2 diabetes mellitus (T2D), it is pertinent to first discuss methodological approaches to study vascular function in humans. Further, while the role of the sympathetic nervous system can be pharmacologically mimicked when investigating the sympathetic contributions to vascular function (1, 2), these studies inherently discount the effect of endogenous sympathetic outflow, which can limit the widespread application of these techniques across populations with markedly different resting sympathetic outflows. Therefore, techniques making use of direct recordings of muscle sympathetic nerve activity (MSNA), whilst also quantifying the ensuing vasoconstrictor responses to this sympathetic outflow might reasonably provide a more holistic view on the 'physiological' role of sympathetic vasoconstriction in vascular function across populations or individuals. Therefore, we will also review methodological approaches which have been developed in order to study the end-organ responses to endogenous sympathetic outflow, a phenomenon commonly referred to as sympathetic transduction. Following these methodological details, we will thoroughly examine existing literature regarding the central and peripheral actions of insulin, and how they may be modified by insulin resistance (IR) or T2D, culminating in presentation of the critical knowledge gaps and experimental hypotheses of the current dissertation.

Assessments of Vascular Function in Human Skeletal Muscle

A multitude of techniques can be applied to the study of vascular function in human skeletal muscle, which have been excellently detailed elsewhere (3, 4). The at present, gold-standard methodology to probe the mechanisms of vascular function is to locally (i.e., arterially) administer an agonist or antagonist of a given receptor/enzyme that would produce one or more vasoactive substance(s). The vascular effect of these infusions is then most commonly quantified by measuring changes in blood flow via thermodilution, venous occlusion plethysmography, Duplex

Doppler ultrasound, or other forms of advanced imaging (e.g., magnetic resonance or positron emission tomography). Generally, intra-arterial infusions are considered gold-standard because they largely avoid the confounding effects of these vasoactive substances on systemic hemodynamics, and acid-base or fluid balance. Therefore, a distinct advantage of this method is that it allows interrogation of the vascular-specific effects of these compounds. Yet, there are two important drawbacks to this technique. First, the invasive nature of arterial line placement largely limits its wide-spread applicability in human research, and confers some additional risk. Second, and perhaps more important to the present discussion, these studies only represent the effect of luminally administered compounds, therefore the kinetics as well as physical properties (e.g., lipophilicity) of the drug may determine the concentration that reaches the interstitial space, putatively altering the efficacy of the blockade, or the receptor populations blocked (e.g., endothelial cell versus vascular smooth muscle cell receptor activation). Figure 1 provides a Doppler ultrasound image obtained during intra-arterial infusion of a vaso-active substance, and also depicts pertinent parameters for appropriate quantification of arterial flow when using Doppler.

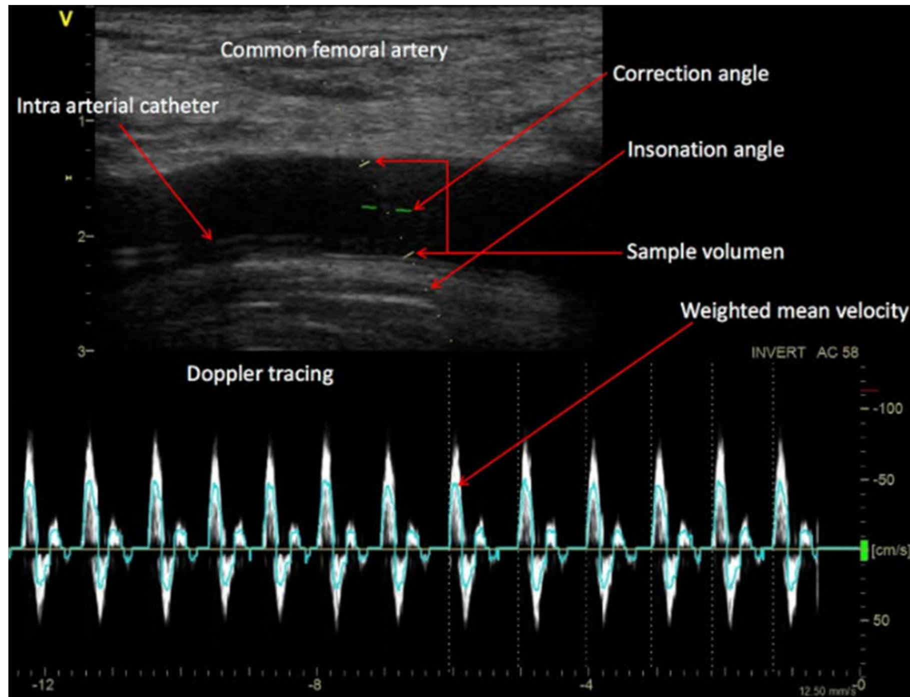

Figure 1. Image of ultrasound Doppler tracings from the common femoral artery. Note the arterial catheter for drug infusion and/or arterial blood pressure measurements. Insonation angle, correction angle, and sample volume are of great importance for a valid determination of arterial flow. The blue line in the Doppler Tracings is weighted mean blood velocities, i.e., a weighted average of the sample volume. Adapted from Gliemann et al. *European Journal of Applied Physiology*, 2018.

In contrast to intra-arterial infusion, another methodology to probe vascular function, is to induce local ischemia (typically of the forearm) via supra-systolic arterial cuff occlusion for ~5 minutes. A substantial increase in blood flow occurs during the reflow phase of this test (i.e., reactive hyperemia), and the flow-induced increase in sheer stress along the vessel wall causes endothelium-dependent vasodilation (primarily nitric oxide-; NO-mediated) of the conduit artery (5, 6). The magnitude of the upstream conduit vessel vasodilation (typically the peak change in vessel diameter following the occlusion) occurring ~60 seconds post-initiation of reflow can then be taken as an index of conduit artery (macrovascular) function (3, 6). Further, the reactive hyperemia can be quantified in a variety of ways (peak versus total area under the curve, etc.), and taken as an index of resistance vessel (microvascular) function. It is prudent to note that the reactive hyperemia response is likely not an endothelium-dependent phenomenon, but rather due

to a myriad of local mechanisms which putatively stem from the build-up of metabolic vasodilators during the ischemic period. Mechanistically, peak reactive hyperemia is mediated predominantly (~50%) by inward rectifying potassium channels, while the total area under the curve response is mediated near-entirely (~90%) by inward rectifying potassium channels and sodium/potassium-ATPase (Na^+/K^+-ATPase) (7). While the distinct molecule(s) responsible for activation of these channels during transient ischemia are not known, K^+, adenosine triphosphate (ATP), bradykinin, hydrogen peroxide (H_2O_2) and epoxyeicosatrienoic acids may all be involved (8). Therefore, this test yields two distinct indices of vascular function, one primarily endothelium-dependent, and the other more likely endothelium-independent, with varying mechanisms of action.

While non-invasive, this technique is subject to large inter-individual variability, perhaps related to the variability in the magnitude of ischemia evoked or as a functional effect of baseline diameter (9). Although, differences in resting oxidative metabolism and skeletal muscle fiber type may also plausibly contribute. Finally, important to these studies, while the shear stress-induced increase in vessel diameter is considered primarily NO-mediated, it is not exclusively mediated by NO and there are heterogeneous pathways underlying flow-mediated dilation between populations (10). Therefore, a distinct drawback to this technique is the limited generalizability of the results to mechanistic underpinnings of the dysfunction. Two other distinct paradigms to test vascular function have been developed in recent times, namely assessments of the blood flow responses to single limb exercise or to passive limb movement. Similarly, changes in vessel diameter and/or changes in hyperemia can be used as indices in these instances. These methods carry similar limitations to ischemia-induced assessments of vascular function, each containing NO-components, but being affected by a diverse array of other mechanisms. Therefore, a common strategy employed in these types of investigations is to administer oral or intravenous

medication(s) to probe the role of specific pathways in the ischemic-, exercise- or sheer- mediated impairments in vascular function across populations (3). Yet important to these methods, systemic administration of drugs carries with it inherent effects on other vascular and non-vascular systems.

Assessments of Sympathetic (Vascular) Transduction in Humans

Direct recordings of the human sympathetic nervous system have been reported since their development in Sweden in the late 1960s (11, 12), via a technique called microneurography. Simply, microneurography involves insertion of a tungsten microelectrode into a peripheral (e.g., peroneal or radial) nerve. The microelectrode is then guided into sympathetic fascicle(s) within that nerve containing efferent C-fibers that innervate the vasculature of muscle. This direct neural signal is connected to a high-gain recording amplifier and signal-processed (i.e., band-pass filtered, rectified and integrated) to provide a sympathetic neurogram which contains “bursts” (phasic discharges) of sympathetic outflow. The primary advantage to these invasive assessments of human sympathetic outflow is that they have absolute resolution, and thus are temporally more accurate than other assessments such as measuring plasma norepinephrine.

For the last half-decade microneurography has been used extensively to study the generation and regulation of central sympathetic outflow in human health and disease. Indeed, it is now appreciated that sympathetic overactivity accompanies the natural aging process as well as a multitude of disease states (13-15). In this regard, sympathetic overactivity is associated with a host of deleterious consequences, yet most striking is the positive association between elevated sympathetic outflow and poor prognosis in both community-dwelling elderly individuals (16) as well as those with overt cardiovascular disease (15, 17, 18). However, while the regulation of sympathetic outflow and its deleterious consequences have been well characterized, microneurographic assessments simply provide characterization of sympathetic neural outflow

and thus do not provide any quantitative information regarding the ensuing sympathetically-mediated vasoconstriction.

Fundamentally, in order for muscle sympathetic nerve activity (MSNA) to appropriately regulate vascular tone, MSNA must be converted (transduced) into an end-organ response (i.e., sympathetic vascular transduction). This requires the neural signal to successfully initiate the release of neurotransmitter(s) into the synaptic cleft, resulting in abundant binding of neurotransmitter(s) to their target receptor(s), wherein the rate of receptor binding must exceed the rate of removal of neurotransmitter from the synaptic cleft (i.e., neuronal reuptake). In total, this net positive binding must then result in wide-spread signal transduction within vascular smooth muscle, culminating in vasoconstriction. Further, for BP regulatory purposes, the local vasoconstriction must be sufficient enough to then be translated into an appreciable increase in BP (sympathetic transduction to BP). The intricacy of this system and its feedback regulator(s) is best appreciated when the relationship between resting MSNA and resting BP is examined. Indeed, there is a well-documented dissociation between resting MSNA and blood pressure (19-21), suggesting that alterations in any number of these components to signal transduction may occur across individuals or along the disease spectrum, thereby modifying the relationship between MSNA and the magnitude of the resulting vasoconstriction.

Therefore, the development and application of techniques to assess sympathetic vasoconstrictor responsiveness are imperative in order to more completely understand the functional effect of MSNA on the peripheral vasculature. In this regard, a common approach is to intra-arterially infuse an α-adrenergic agonist, to mimic the action(s) of efferent sympathetic outflow and induce vasoconstriction. A known quantity of agonist can be administered, and the vascular response quantified across conditions or experimental groups [e.g., (22)]. However, a

critical limitation to the majority of studies examining vasoconstrictor responsiveness to pharmacological stimuli is that they do not have concurrent direct recordings of MSNA, so the level of perceived agonist sensitivity is not understood with respect to the amount of endogenous neural activity. This is important because resting MSNA is inversely related to the vasoconstrictor response to intra-arterial infusion of 1) exogenous norepinephrine and 2) tyramine to induce endogenous norepinephrine release in young healthy subjects (23). Further, the inverse correlation between resting MSNA and vasoconstrictor response to exogenous norepinephrine exists in young, but not older men (24). Cumulatively, these data support the notion that the large variability in resting MSNA in healthy individuals may, in part, be accounted for by the magnitude of ensuing vasoconstriction, and that this relationship can be modified (i.e., lost) by cardiovascular risk factors (e.g., age). In total, these studies demonstrate the distinct benefit to simultaneously quantifying endogenous sympathetic outflow, as well as the magnitude of conferred vasoconstriction.

To this end, a number of investigative approaches have been applied using microneurography, to quantify both sympathetic outflow (i.e., MSNA) as well as the amount of vasoconstriction conferred for a given amount of MSNA (sympathetic transduction). Experimentally, two distinct paradigms have been developed to study sympathetic transduction in humans. The first paradigm involves evoking reflex sympatho-excitation and quantifying the change in MSNA and change in vasoconstriction (indexed via vascular conductance or resistance) or BP (sympathetic transduction during stress). The second paradigm involves employing a mathematical model/analytical approach in order to quantify the vasoconstrictor or pressor responses to spontaneous bursts of MSNA at rest (sympathetic transduction at rest).

Sympathetic Transduction During Stress

Several different sympathetic stimuli and methodological approaches have been developed to quantify sympathetic transduction during reflex sympatho-excitation. Generally, the aforementioned sympathetic stressors can be split into three distinct categories 1) handgrip exercise (25-30); 2) cold pressor test (31); 3) orthostatic stress (32, 33). The general premise of these investigations, as noted above, is to evoke a given change in MSNA and observe the ensuing change in vascular conductance or resistance. Yet, a myriad of approaches to quantifying sympathetic transduction during stress have been applied, including simple “delta-delta” calculations, or the ratio of the change in vasoconstriction per change in MSNA (27), linear regression of bins of MSNA and BP or vascular conductance/resistance (25, 34), and a modified Poiseuille’s Law transfer function analysis (30). An example of the linear regression quantification method is provided in Figure 2, which depicts the transduction slope in a single subject derived from linear regression of bins of MSNA and vascular resistance at baseline and during static handgrip exercise, as presented in the original investigation by Halliwill et al. (25).

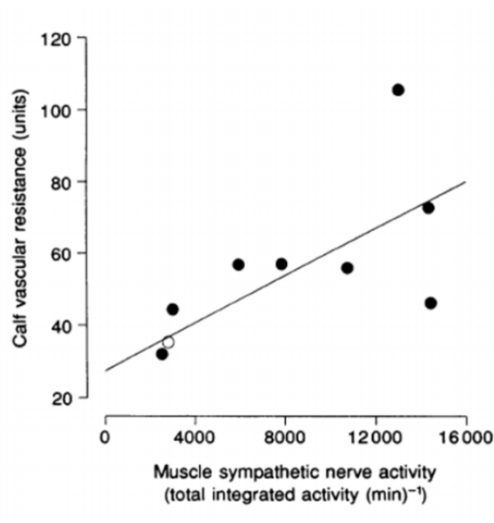

Figure 2. Representative transduction relation derived from one subject (Panel A). Open circle, 5 min baseline that preceded handgrip exercise. Closed circles, the mean nerve activity and vascular resistance during a 30 sec period. Regression: calf vascular resistance (CVR)= 27.4 + 0.0033 MSNA; r, 0.78. Adapted from Halliwill et al. *journal of Physiology*, 1996.

Important for the present discussion, there are substantial limitations to these techniques. First, alternative methods for quantifying the vascular outcome can dramatically change perceived results (29). This is likely because of the non-linearity between vascular conductance and vascular resistance, which is best described by the impact of this non-linearity on published results related to sympathetic vasoconstriction during exercise (35-37). Collectively, these discussions support the use of percent changes in vascular conductance as a primary outcome measure when assessing sympathetic vasoconstriction based upon the following principles: Under conditions of constant perfusion pressure, changes in vascular conductance more accurately reflect true vasoconstriction (changes in blood flow) than vascular resistance (36). Further, the same theoretical percent increase in radius always induces the same percent increase in vascular conductance (but not absolute value increase), independent of the prevailing magnitude of blood flow (37). Taken together, these principles indicate that percent changes in vascular conductance most accurately represent sympathetic vasoconstriction under conditions where blood flow changes (e.g., from rest to stress).

The apparent conclusion above would seem trivial with respect to the use of these stressors. *"Why not simply calculate percent changes in vascular conductance?"* Unfortunately, that quantification method can lead to spurious results. Indeed, some of these stressors (e.g., handgrip or cold pressor test) evoke large increases in BP, which are undoubtedly partially mediated by peripheral sympathetic vasoconstriction, but also likely contain cardiac output components (38, 39). Independent of the mechanism, the large increase in BP represents a competing force for the regulation of blood flow. Indeed, the increase in BP favors increases in blood flow, which would tend to increase vascular conductance, while the increase in MSNA would tend to reduce vascular conductance. In some instances, the increase in BP is substantial enough to cause an overt increase

in blood flow (39, 40) to skeletal muscle during these stressors. Therefore, in these cases, calculation of sympathetic transduction would yield the following erroneous conclusion: Increases in MSNA elicit increases in vascular conductance (i.e., vasodilation). Stated simply, for all stressors involving changes in BP or cardiac output, the inter-relationship between BP, MSNA, and blood flow/vascular conductance likely leads to erroneous under-estimation of local sympathetic vasoconstriction if left unaccounted for (30). This may be a perceived benefit to the modified Poiseuille's Law model, which accounts for BP-driven changes in blood flow, or the use of orthostatic stressors (particularly lower body negative pressure) that largely avoid changes in BP.

Another key issue with these methodologies is that they largely discount the vasoconstrictor effect of resting sympathetic outflow, and thus interpreting the results of super-imposing reflex sympatho-excitation between conditions with different resting sympathetic outflows (See chapter 4) can be challenging. Moreover, the mass increase in neurotransmitter release during stress likely alters several aspects of neurotransmitter kinetics. Indeed, inducing sympatho-excitation might reasonably alter the presence/magnitude of co-transmission (e.g., adenosine triphosphate or neuropeptide Y release), the rate of neuronal reuptake, the rate of spillover of norepinephrine into the circulation and/or conversely the rate of norepinephrine uptake by the tissue from the circulation, and neurotransmitter-receptor binding equilibriums. Therefore, physiologically, the conclusions drawn from investigations into sympathetic transduction during stress should not necessarily be extrapolated to the resting condition.

Sympathetic Vascular Transduction under Resting Conditions

Sympathetic outflow at rest is considerably less than during the stressors outlined above, and to date, two distinct methodological approaches have been developed to study sympathetic

transduction at rest. The first technique is a spike-triggered averaging methodology originally developed by Wallin and Nerhed (41). The second is a methodology developed more recently by Briant and colleagues (42) which employs linear regression analysis of binned MSNA burst area against diastolic BP.

In 1982, Wallin and Nerhed (41) applied a signal-averaging technique analogous to spike-triggered averaging in order to better understand the magnitude and temporality of the effect of spontaneous fluctuations in MSNA on BP and the transient shortening of R-R interval. Original records from this seminal investigation by Wallin and Nerhed (41) are provided in Figure 3. In this model, every burst of MSNA acted as a trigger (event), and was subsequently followed for 15 cardiac cycles. The change in BP and reduction in R-R interval was then calculated as the instantaneous BP/R-R interval value in each of the 15 cardiac cycles minus the value in the cardiac cycle in which the burst originated. This procedure was repeated for all cardiac cycles associated with a burst of MSNA over the 3-minute period, and the responses were then averaged to provide a mean change in BP/R-R interval for all the bursts of MSNA.

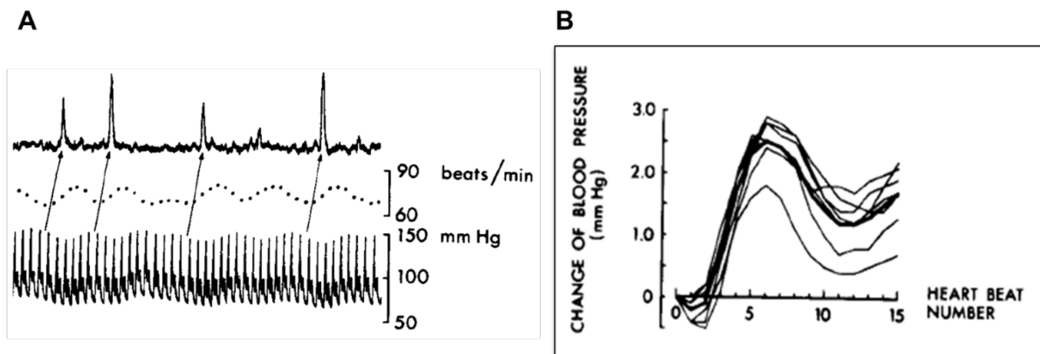

Figure 3. (Panel A) Example of the relationships between spontaneous variations of sympathetic muscle nerve activity (upper Trace), instantaneous heart rate (middle trace), and blood pressure (bottom trace) in a representative subject. Each arrow in A indicates the baroreflex latency (approximately 1.3 s) and shows during which heart beat the sympathetic burst was initiated. (Panel B) Changes of diastolic blood pressure following peak of sympathetic impulses (occurring at heart beat 0) during the rest period. Adapted from Wallin and Nerhed *Journal of the Autonomic Nervous System*, 1982.

This technique has been significantly advanced and validated by investigations in our laboratory (43-45). Indeed, our laboratory extended this work to include blood flow measurements so that local vasoconstrictor effects of MSNA could be quantified (43), but has also quantified the cardiac output responses (unpublished data) following bursts of MSNA. Cardiac output increases across the first 2-3 cardiac cycles, yet returns to baseline values after cardiac cycle 4 thus having little to no influence on the peak BP or vascular conductance responses. Further, the extension of this technique to local vasoconstrictor responses subsequently enabled our laboratory to verify the sympathetic origin of these reductions in vascular conductance. Indeed, using intra-brachial infusion of saline (control), phentolamine (non-selective α-adrenergic receptor antagonist) and phentolamine with co-infusion of angiotensin II (to negate the effect of the increase in blood flow during phentolamine administration) our laboratory demonstrated that the reduction in vascular conductance following bursts of MSNA was almost entirely abolished by α-adrenergic blockade (45). The summary data for this investigation is presented in Figure 4.

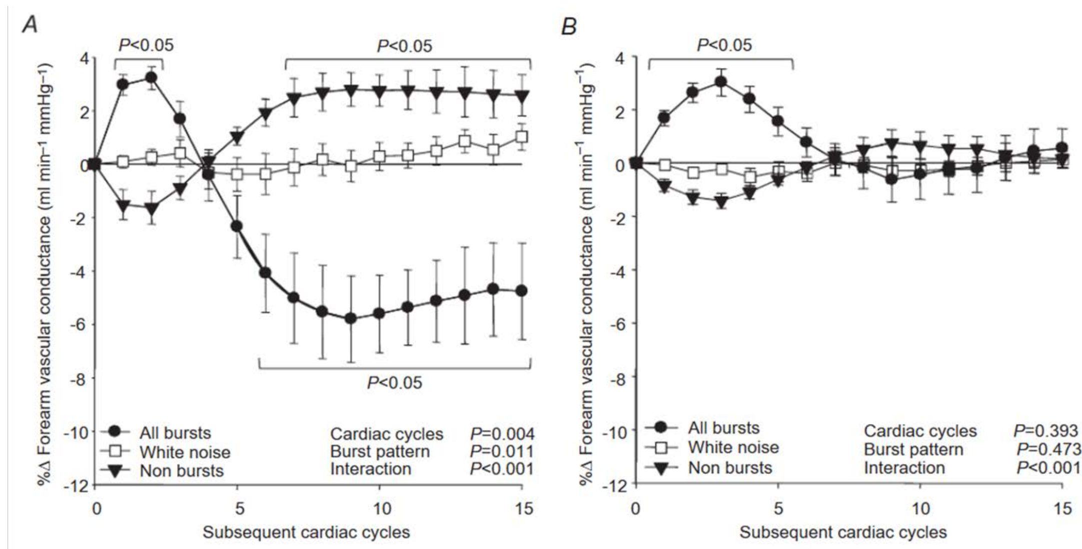

Figure 4. Summary data of beat-by-beat percentage changes in forearm vascular conductance following all spontaneous muscle sympathetic nerve activity bursts, non-bursts, and white noise during saline infusion (A) and during phetolamine + angiotensin II co-infusion (α-adrenergic blockade; B). Brackets denote significant differences from percentage changes in white noise. Adapted from Fairfax et al. *Journal of Physiology,* 2013.

Separately, Briant et al. (42) developed another analytical technique to examine sympathetic transduction to BP. In that investigation, sympathetic transduction was quantified as the weighted linear relationship between diastolic BP and MSNA burst area binned in 1% sec bins. For this linear regression, each diastolic BP was associated with the MSNA burst area within a 2-cardiac cycle window from 6-8 cycles preceding the diastolic BP (i.e., the inverse of the relationship commonly used to investigate baroreflex sensitivity). In other words, a given DBP was traced back 6–8 cardiac cycles to investigate the influence of MSNA on DBP. This window was selected based on previous data (30, 46) examining the time to peak sympathetic effect, and was verified by conducting the same analysis for all box regions (2 cycle windows) encompassing up to a 10 cardiac cycle lag. In all instances, the 6-8 cardiac cycle lag provided the greatest measure of sympathetic transduction. This analytical technique was also completed for total peripheral resistance in place of diastolic BP. Although, to date, no follow-up investigations using this technique have been published.

Indices of Sympathetic Transduction: Which to use?

As outlined above, a wide variety of methodologies have been applied to the study of sympathetic transduction in humans, both at rest, and during reflex sympatho-excitation. However, the level of reflex sympatho-excitation evoked likely alters several aspects of neurotransmitter release and kinetics, suggesting that these investigations may be more relatable to periods of extreme sympathetic neurotransmission, rather than to resting physiology. In that regard, investigations examining sympathetic transduction during orthostasis may be more applicable to the "every-day" phenomenon of sympathetic transduction, as humans spend a large portion of the day in the upright position. Yet, all of these approaches (i.e., assessments of sympathetic transduction during stress) inherently discount the vasoconstrictor effects of resting sympathetic

outflow, and examination of sympathetic transduction during stress between conditions or groups with markedly different resting sympathetic outflows are necessarily impeded by this omission. Therefore, quantifications of the vasoconstrictor responses to spontaneous bursts of sympathetic outflow offer a unique opportunity to study sympathetic transduction in the 'normal' physiological state.

Finally, and perhaps the most perplexing of all, despite widespread use and adoption of these techniques, none of these methodologies with the exception of signal-averaging quantifications of beat-to-beat sympathetic transduction have been experimentally verified to represent sympathetic vasoconstriction (responses mediated primarily by adrenergic receptors). Based on the above description and discussion of methodological and physiological limitations associated with these techniques, the signal-averaging quantification of resting sympathetic transduction may be considered the most resolute, and robust approach to study sympathetic transduction in humans. Although, it should be noted that when sympathetic transduction is studied across populations with drastically different sympathetic outflows, study designs incorporating blockade of α- and β- adrenergic receptors to observe the change in basal blood flow may be requisite to quantify resting sympathetic transduction.

Insulin Receptor Signal Transduction: General Features

The insulin receptor is a ligand-activated, membrane-spanning tyrosine kinase. Yet, the downstream molecular signaling pathways that determine the function of insulin signaling are complex, and tissue-specific. Generally, insulin receptor activation is coupled to signal transduction pathways via a family of adaptor molecules, conveniently named insulin receptor substrates (IRS). In the periphery, IRS-1 is essential to the biological action of insulin (discussed further below). On the other hand, IRS-1 is expressed diffusely through the central nervous system

(47), but is generally not concentrated in areas of the hypothalamus which contain the highest densities of insulin receptors (47, 48). In support of the notion that IRS-1 does not play a large role in mediating the central actions of insulin, (complete) knockout of IRS-1 in animals results in glucose intolerance (presumably due to impaired peripheral actions of insulin), but does not necessarily produce the traditional phenotype of abnormal energy homeostasis that is typically attributed to alterations in central insulin signaling (49, 50). These seminal findings (49) were associated with the discovery of IRS-2. Indeed, IRS-2 in the central nervous system appears to be integral for the central metabolic actions of insulin (51, 52), and mice lacking hypothalamic IRS-2 exhibit abnormal feeding, indicating IRS-2 likely plays an integral role in mediating the energy homeostatic mechanisms of central insulin signaling (53, 54). Little is known regarding the specific role of IRS-2 in insulin-induced sympatho-excitation. Despite the central and peripheral actions of insulin being mediated *primarily* by different members of the IRS family, both appear to exert their functional effects through phosphatidylinositol-3 kinase (PI-3k), protein kinase B (Akt) signal transduction pathways (48).

Insulin Signaling in the Central Nervous System: Implications for sympatho-excitation

The majority of literature has focused on the actions of insulin within the hypothalamus, however, anatomically insulin receptors are present in both hypothalamic and extra-hypothalamic areas, and these receptor sub-populations are capable of influencing metabolic and cardiovascular function profoundly (55). Key functions of central insulin signaling include the regulation of appetite, nutrient metabolism, energy homeostasis, and recently cognition as well as reproduction (48, 54, 56, 57). Yet, in addition to these metabolic and behavioral effects, insulin signaling within the central nervous system has well-documented cardiovascular effects. To date, the central neural signaling pathways mediating the cardiovascular effects of central insulin signaling remain

incompletely understood, although glucocorticoids and neuropeptide Y are likely involved (48, 58).

In humans, both acute carbohydrate ingestion (59, 60) and pharmacologically induced hyperinsulinemic-euglycemia (61-66) are associated with significant increases in sympathetic outflow. Importantly, this sympatho-excitatory response to hyperinsulinemia in humans appears to be selective to muscle, as hyperinsulinemia has little effect on skin sympathetic outflow (61). These data are complimented by rodent studies, documenting an increase in lumbar, but not renal or adrenal sympathetic outflow during hyperinsulinemia (67, 68) further supporting a highly selective sympatho-excitation directed to skeletal muscle. Original neurograms demonstrating the marked sympatho-excitation in humans during elevations in plasma insulin, induced by both consumption of a mixed meal and hyperinsulinemic-euglycemic clamp methods are provided in Figure 5.

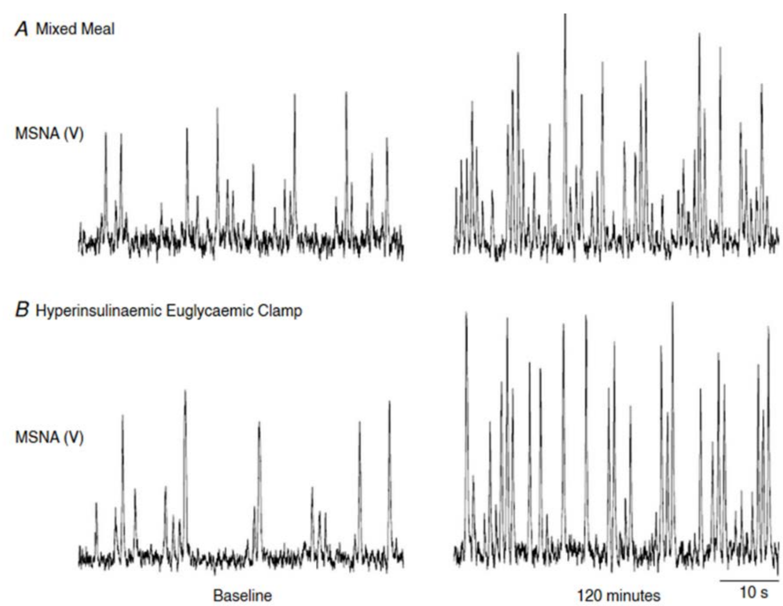

Figure 5. Original records illustrating muscle sympathetic nerve activity at baseline and at 120 min following a mixed meal (A) and during the hyperinsulinemic euglycaemic clamp (B). V, volts. Adapted from Young CN et al. *Journal of Physiology*, 2010.

A series of investigations aimed at determining the source(s) of this sympatho-excitatory response during acute hyperinsulinemia have been carried out. One line of evidence aimed to determine if insulin per se was the main sympatho-excitatory stimulus, or rather, if the stimulus might be carbohydrate metabolism. To answer this question, Vollenweider and colleagues (64) utilized an experimental design to disentangle hyperinsulinemia from the stimulation of carbohydrate metabolism, using three experimental paradigms: glucose infusion, fructose infusion, or combined insulin/glucose infusion. Despite similar increases in carbohydrate oxidation between conditions (a function of glucose/fructose infusion rate being functionally matched across trials), fructose infusion had little effect on insulin release, while glucose infusion moderately increased plasma insulin concentration, and insulin/glucose co-infusion markedly increased plasma insulin concentration. Fructose infusion failed to stimulate any increase in MSNA, while a dose-dependent stimulatory effect of insulin on MSNA was observed in the other two conditions. These data indicate that carbohydrate metabolism itself, plays little role in post-prandial sympatho-excitation.

The other line of investigation sought to determine whether or not the sympatho-excitatory actions of insulin were due to a direct action of insulin in the central nervous system, or whether the vasodilatory actions of insulin and the subsequent (albeit minor) reductions in arterial blood pressure during hyperinsulinemia (63, 66, 69, 70) may have stimulated a baroreceptor-mediated increase in sympathetic outflow. Contributing to the latter hypothesis, was the observation of a large dissociation between the time of peak insulin concentration and the subsequent (delayed) peak sympatho-excitation during hyperinsulinemic-euglycemic clamp studies (62, 64). Despite this apparent dissociation, several lines of evidence suggest that the sympatho-excitation is indeed mediated by central insulin stimulation. First, kinetically, the change in lymph insulin concentration, as an index of interstitial insulin concentrations, more closely reflects the time-

course of sympatho-excitation during hyperinsulinemia, suggesting that the aforementioned dissociation is related to the delay between peak plasma and peak interstitial concentrations of insulin (71). Second, in animal models micro-doses (doses lacking a systemic effect) of insulin administered intracerebroventricularly increase lumbar sympathetic outflow (67). Further, a follow-up study in humans conducted on the basis of those animal data demonstrated that moderate elevations in plasma insulin, which are insufficient enough to elicit peripheral vasodilation, still increase MSNA in humans (72). Collectively, these data provide strong evidence that the sympatho-excitation during hyperinsulinemia is due to a direct effect of insulin within the central nervous system. In order to further understand these central actions of insulin, Scherrer and colleagues (62) examined the effect of dexamethasone, a corticosteroid that modulates corticotropin releasing hormone and neuropeptide y release, on insulin-induced sympatho-excitation. In that investigation, dexamethasone administration markedly attenuated the sympathetic effect of insulin, suggesting these may be key underlying signaling pathways (62). Alternatively, dexamethasone administration may inhibit the transport of insulin into the central nervous system (73), thereby indirectly attenuating the sympathetic effect of insulin.

In addition to increasing sympathetic outflow to skeletal muscle, insulin has been suggested to enhance arterial baroreflex function. In animal models lateral ventricular infusion of insulin augments the gain of the arterial baroreflex control of both heart rate and lumbar sympathetic nerve activity (74). In contrast, insulin does not appear to greatly affect cardiac baroreflex sensitivity in humans (66, 75), but clearly enhances the gain of the arterial baroreflex control of MSNA, both post-prandially and during pharmacologic hyperinsulinemic-euglycemia (66). Taken together then, in addition to stimulating sympathetic outflow, insulin also enhances the gain of the arterial baroreflex control of sympathetic outflow in both animals and humans.

Vascular Insulin Signaling: Molecular Mechanisms

As briefly presented above, vascular insulin signaling is associated with phosphorylation of IRS-1. Phosphorylation of IRS-1 leads to activation of PI-3k, which through the generation of lipid products initiates a cascade of serine kinases where phosphoinositide-dependent kinase-1 (PDK-1) is phosphorylated and subsequently activated. PDK-1 phosphorylation then activates and phosphorylates Akt, which through a variety of downstream effectors will result in the pleiotropic effects of insulin signaling. Importantly, this PI3k-Akt pathway is the same pathway that when activated elicits the metabolic actions of insulin in skeletal muscle, suggesting tight coupling of the vascular and metabolic actions of insulin (76). Specifically, Akt activation of downstream effectors results in 1) phosphorylation of endothelial NO synthase, which will stimulate the production of NO (potent vasodilator) and 2) the induction of glucose transporter type-4 (GLUT-4) directly within skeletal muscle. However, a countercurrent pathway also exists wherein insulin may stimulate a Ras/mitogen-activated protein kinase (MAPK) dependent pathway that 1) stimulates the endothelial production of the potent vasoconstrictor peptide, endothelin-1 (ET-1) and 2) may also stimulate growth and mitogenesis. Functionally, these signal transduction pathways are distinct and do not involve the same molecular mediators (76). For example, the PI3k inhibitor, wortmannin, does not alter ET-1 secretion by cultured endothelial cells (77). Functionally though, it does appear that crosstalk between these pathways may exist, as endothelium-derived NO production has been reported to limit endothelin-1 secretion (78, 79). However, ET-1 production does not exclusively induce vasoconstriction. Indeed, there are two distinct types of ET-1 receptors, which elicit differential actions based on the subpopulation of receptors activated. Activation of ET-1 (ETA) receptors located on vascular smooth muscle produces potent vasoconstriction, while activation of ET-1 B (ETB) receptors elicit either

vasoconstriction (vascular smooth muscle ETB receptors) or vasodilation (endothelial ETB receptors) based on location (80).

In vitro, the vasoactive actions of insulin are mediated solely through these two pathways, with the net effect typically being vasodilation (76, 81, 82). These findings are mirrored in vivo, wherein hyperinsulinemia is accompanied by a potent skeletal muscle vasodilation in healthy humans (69, 83, 84). The vasodilatory response to insulin appears to be dose-dependent (69, 85), however there is data to suggest that this effect may also be time-dependent (65, 86). Although, some of the apparent time-dependency may actually be a factor of the two-distinct phases of insulin-induced hyper-perfusion. In rats, increases in microvascular perfusion (assessed via contrast enhanced ultrasound; CEU) occur within minutes (87) and precede overt changes in limb blood flow (88). Similarly, in humans, the time-course of CEU-derived microvascular perfusion measurements generally suggest a microvascular effect of insulin occurs prior to the macrovascular vasodilation during hyperinsulinemia (69, 89). These studies have attributed the microvascular effects of insulin to increasing the uniformity of capillary perfusion. In general agreement with the physiological phenomenon, but contrasting with respect to mechanism, a recent study using intravital microscopy, CEU, glucose dispersion and muscle glucose uptake derived from radio-tracer methodologies revisited the phenomenon of proposed capillary recruitment. In this investigation, insulin failed to change capillary perfusion, and skeletal muscle glucose uptake during insulin administration appeared to be a function of enhanced microvascular flow velocity and glucose dispersion, rather than capillary recruitment. Further, these investigators provided evidence that CEU-derived measures were more indicative of microvascular flow velocity than capillary recruitment per se (90).

In healthy humans, consistent with the in vitro findings, it appears that the vasodilatory response to insulin is near-exclusively mediated by NO. Indeed, the NO synthase inhibitor N(G)-monomethyl L-arginine (L-NMMA) abolishes the increase in blood flow during hyperinsulinemia (83, 91). In addition, supporting the notion that the Ras/MAPK signaling pathways plays little role, intra-arterial administration of the ET-1 A (ETA) receptor antagonist (BQ-123) does not improve insulin-stimulated blood flow in healthy individuals (92, 93). Metabolically, abolishing the increase in blood flow during hyperinsulinemia impairs skeletal muscle glucose uptake by ~35% (83), suggesting that the vasoactive and metabolic actions of insulin are tightly coupled (see Figure 6).

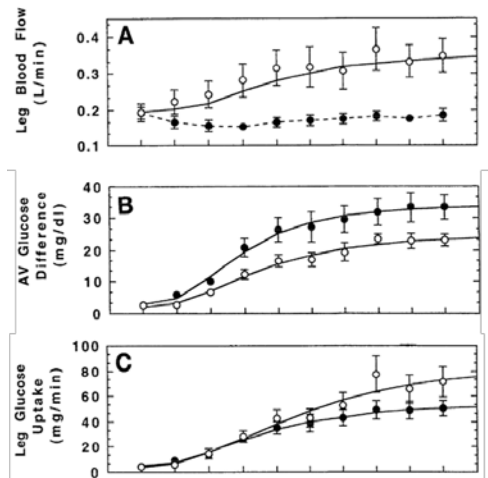

Figure 6. Time course of leg blood flow (A), arteriovenous glucose differences (B), and leg glucose uptake (C) in response to euglycemic hyperinsulinemia alone (open circles) or with superimposed intrafemoral artery infusion of L-NMMA (16 mg/min; closed circles). Solid lines present fitted response curves obtained by MacAllfit. Adapted from Baron et al. *American Journal of Physiology*, 1996.

These data were recently followed-up by a rather invasive human study using arterial and venous catheters to derive arterial-venous glucose difference, interstitial microdialysis to measure interstitial glucose concentration, and Doppler ultrasound to measure blood flow, as well as multiple vasodilator and vasoconstrictor compounds administered intra-arterially. Indeed, with

this invasive design, McConnell et al. (91) were able to calculate functional membrane permeability to glucose in humans, and to demonstrate for the first time, that blood flow was a rate-limiting determinant of interstitial glucose concentration (and thus glucose uptake). Collectively, it is now firmly established that in health, the vasoactive actions of insulin are primarily NO-dependent, and that these actions contribute importantly (~35%) to the glucose uptake response in skeletal muscle during hyperinsulinemia.

Interactions between Vascular Insulin Signaling and Sympathetic Vasoconstriction

An interesting phenomenon within the exercise literature is the notion that during exercise, super-imposing a sympathetic stimulus (e.g., reflex sympatho-excitation via lower body negative pressure; LBNP) has little to no effect on active muscle blood flow. This introduced the concept of a complete 'lysing' of sympathetic vasoconstriction in active muscle during exercise (i.e., functional sympatholysis). Indeed, the endothelium has emerged as an important feedback regulator of sympathetic vasoconstriction, putatively through myoendothelial projections which electrically couple the endothelium and vascular smooth muscle (1). More recent investigations aimed at further understanding the molecular mechanisms of functional sympatholysis have extended this work to invasive pharmacology. As such, it is now well accepted that a variety of endothelial-dependent hyperpolarizing agents, such as acetylcholine and adenosine triphosphate are capable of abolishing sympathetic vasoconstriction (mimicked by phenylephrine infusion; α1-adrenergic agonist) during small muscle mass exercise (1). While the activation of endothelial NO synthase by insulin and subsequent production of NO is distinct, separable, and independent from the classical g-protein coupled receptor interactions which produce NO such as those activated by acetylcholine (94), investigations have been carried out in similar fashion in order to determine if insulin too is capable of attenuating sympathetic vasoconstriction.

Seminal in vitro studies documented an attenuated vasoconstriction in response to norepinephrine in the presence of insulin (95, 96), and this effect appears to be endothelium-dependent as well as require an intact insulin signaling cascade, since both endothelial denudation and PI3k-inhibition abrogate this effect (84). In part based on these in vitro findings, Lembo and colleagues (97) set out to determine if local insulin administration was indeed capable of attenuating sympathetic vasoconstriction in humans. In order to test this, the authors intra-arterially administered insulin and super-imposed reflex sympatho-excitation via graded LBNP. In the presence of insulin there was a blunted increase in forearm vascular resistance during graded LBNP, indicating a sympatho-attenuating action of vascular insulin signaling. These findings were then translated using invasive pharmacology by the same group, to determine that local insulin administration attenuated sympathetic vasoconstriction via a NO-component present in both the α_2- (98) and β- (99) adrenergic signaling pathways (100). Animal investigations from the same group have also verified that this sympatho-attenuating mechanism of insulin signaling occurs via a pathway sensitive to pertussis toxin (101).

Although these findings are universal across investigations from the Italian group (stemming from the labs of Lembo and Trimarco) the observation of a sympatho-attenuating effect of insulin is not unequivocal. Other groups using intra-arterial administration of insulin have both supported (102), and refuted (103) the notion that insulin is capable of inducing local sympatho-attenuation. In addition, there appears to be no difference in the vasoconstrictor response to intra-arterial norepinephrine during systemic (rather than local) hyperinsulinemia (104). Further, the amount of intravenous norepinephrine required to increase diastolic BP by 20 mmHg was significantly less during a hyperinsulinemic clamp at 1 hour, but not at 6-hours post-insulin infusion (105). The lack of a greater pressor reactivity to norepinephrine at 6-hours appeared to be

due to greater plasma clearance of norepinephrine, potentially suggesting a time-dependent effect of this interaction between insulin and norepinephrine signaling. Collectively then, studies have documented attenuated (98, 100, 102), no change in (103, 104), or perhaps even augmented (105) vaso-reactivity in response to exogenous norepinephrine in the presence of insulin. However, almost all of these studies have exogenously administered an agonist to mimic sympathetic nerve activity, and many have used local insulin administration. These methods ultimately limit their ability to account for the inherent sympatho-excitatory features of central insulin signaling (see *Insulin Signaling in the Central Nervous System: Implications for sympatho-excitation).* Therefore, whether or not insulin is capable of locally attenuating its own endogenously-stimulated sympathetic vasoconstriction remains unknown.

Central and Peripheral Insulin Resistance: Implications for Vascular Dysfunction in Type 2 Diabetes

As discussed previously, IR is defined as a reduced tissue responsiveness to insulin stimulation. Indeed, a variety of peripheral and central tissues may display central IR. Therefore, IR may alter the 'normal' physiologic responses to insulin, both centrally (i.e., sympatho-excitation) and peripherally (i.e., insulin-stimulated vasodilation). There is a paucity of literature documenting the alterations in central and peripheral insulin signaling in humans with overt T2D. Rather, the majority of studies in this area have utilized preclinical animal models or human models of IR (e.g., obesity) to infer T2D-associated dysregulation of these pathways. Further, the specific molecular mechanism(s) responsible for these alterations remain multi-factorial and incompletely understood, yet globally, glucotoxicity and hyperinsulinemia via way of upregulated inflammatory cytokines, advanced glycation end products, endoplasmic reticulum stress, oxidative stress, and ceramide may all be involved (in addition to those outlined below).

Central Insulin Resistance: Implications for Sympathetic Outflow

Two distinct animal models of IR have notable investigations examining alterations in the sympathetic nervous system associated with IR. Generally, changes in basal sympathetic outflow appear to be regionally- and model- specific. The first model, the obese zucker rat (OZR), is a model of genetic obesity. In OZR, sympathetic outflow to metabolic organs (e.g., brown adipose tissue) is generally reduced (106, 107), while renal sympathetic outflow to is ~2-fold higher in OZR compared to lean zucker rats (LZR) (108). Importantly though, sympathetic outflow to other vascular beds (e.g., skeletal muscle) remain largely understudied in OZR. Interestingly, OZR also exhibit an increased baseline BP, however, the greater BP in OZR seems to be independent of sympathetic neural activation. Indeed, both ganglionic blockade (109) and combined (systemic) α_1- and β-adrenergic blockade (110) induce similar depressor effects in OZR compared to LZR, indicating that the elevated BP in OZR is not a sympathetic effect. Collectively, these data do not support the notion that OZR display generalized sympatho-excitation. However, it is important to note that these phenomena may be dependent upon the strain of OZR. Indeed, there are several strains of OZR which exhibit a wide range of fasting blood glucoses, ranging from modest to severe hyperglycemia. Indeed, OZR rats with severe hyperglycemia (often called diabetic OZR) exhibit a greater depressor response to hexamethonium (ganglionic blockade) compared to LZR (111), suggesting that sympatho-excitation may occur in this model of genetic obesity in a dose-dependent manner based upon the magnitude of prevailing hyperglycemia. In these animal models (OZR and diabetic OZR), the effect of IR per se on the central sympatho-excitatory features of insulin have not been explicitly assessed.

The second major animal model of IR is a diet-induced obesity (DIO) model (i.e., high-fat diet). Importantly though, this animal model, offers a unique advantage to study the effect of

obesity explicitly because the high-fat diet fed rats diverge into subpopulations following ~4 weeks of dietary intervention. Indeed, some of the rats, defined as obesity-prone (OP) experience large increases in weight (defined as top-third tertile) and develop a hyperinsulinemic-hyperglycemic phenotype. While other rats, termed obesity-resistant (OR; bottom tertile of weight gain) display relatively similar glucose and insulin profiles to control animals on normal chow diets. These sub-populations of animals allow for within-litter comparisons of the effect of obesity. To our knowledge, no investigations have quantified the basal change in sympathetic outflow between OP and OR subpopulations, however other high-fat feeding protocols generally result in an increase in resting lumbar sympathetic outflow (112, 113). Yet, unlike the OZR model, the central sympatho-excitatory effects of insulin administration have been examined in OP and OR DIO rats. Interestingly, intracerebroventricular infusion of insulin evokes a 2-3 fold greater increase in lumbar sympathetic outflow in OP animals, while OR animals exhibit similar lumbar sympathetic responses to insulin as normal chow control animals (114, 115), and this effect in OP animals is abrogated by the blockade of neuropeptide Y (Y1) receptors within the paraventricular nucleus (114). In total, investigations in OZR and DIO rats suggest sympatho-excitation is not a general feature of genetic obesity, however, diet-induced obesity is associated with greater basal lumbar sympathetic outflow, as well as a hyper-sensitivity to central insulin administration. The hyper-sensitivity to insulin administration in OP-DIO rats is demonstrated in Figure 7.

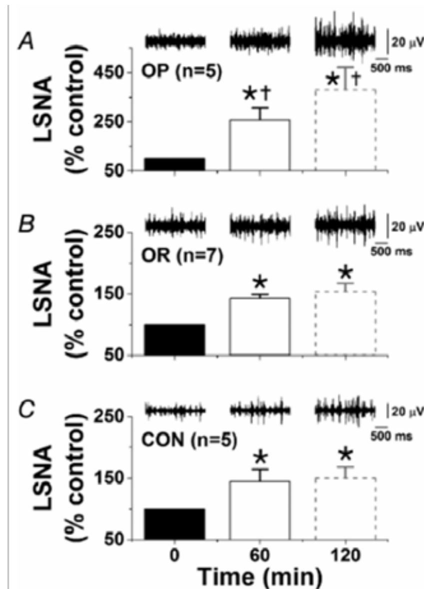

Figure 7. The sympathoexcitatory response to intracerebroventricular insulin is amplified in obesity prone rats (OP) compared to obesity resistance (OR) or control (CON) rats. Note the different scales in OP versus OR/CON. * $P<0.05$ compared to baseline (time zero) within group. † $P<0.05$, OP different from OR and/or CON at the same time point. Adapted from Shi et al. *American Journal of Physiology*, 2019.

Several investigations have transitioned these basic physiological experiments to investigations in human forms of T2D, IR, and their associated co-morbidities (e.g., obesity or metabolic syndrome). Despite marked hyperinsulinemia, measures of resting sympathetic outflow in patients with T2D have yielded equivocal results, wherein some report markedly augmented (116, 117), yet others no difference (118, 119) in resting MSNA in patients with T2D. However, the effect of insulin on central sympatho-excitation in patients with T2D has never been comprehensively examined. Instead, data from obese, IR, and metabolic syndrome patients will have to inform our conclusions on the matter. Interestingly, in obese humans the increase in MSNA during hyperinsulinemia is essentially absent (120). Similar findings are observed following carbohydrate ingestion (121) in obese, metabolic syndrome patients. Importantly, it appears that IR itself directly contributes to this blunted sympathetic response to carbohydrate ingestion. Indeed, obese metabolic syndrome patients with IR exhibit little to no increase in MSNA, while

similar patients whom are relatively insulin sensitive still exhibit some level of sympatho-excitation following oral glucose ingestion (121). Further, a 3-month hypocaloric diet is capable of partially reversing the attenuated sympathetic response to glucose ingestion in obese, metabolic syndrome patients with IR, but not their insulin sensitive counter-parts (122). In total then, these human investigations provide evidence that T2D patients do not necessarily exhibit augmented resting sympathetic outflow, however, the central neural responsiveness to hyperinsulinemia appears to be impaired in obese humans, and IR seems to directly contribute to this impairment.

Yet, MSNA represents only one aspect of sympathetic control of the vasculature, which alone does not account for the magnitude of ensuing vascular smooth muscle contractile response, and ultimately the BP responses to MSNA. In this regard, it is plausible that independent of sympathetic outflow per se, patients with T2D exhibit augmented vasoconstrictor responses to MSNA (i.e., sympathetic transduction). Stated simply, patients with T2D might still exhibit augmented sympathetic vasoconstriction despite similar resting sympathetic outflows compared to control subjects. In general agreement with that notion, patients with T2D exhibit augmented vasoconstriction in response to intra-arterial norepinephrine infusion (123), which may suggest an upregulation in α-receptor sensitivity in T2D.

Peripheral Vascular Insulin Resistance

Animal models of IR are associated with a shift in the balance of production of NO versus ET-1 by peripheral insulin receptor activation. A key feature to IR is that this impairment may be pathway specific (i.e., selective IR). For example, the PI3k-Akt pathway may be impaired with maintained or 'normal' signaling through the Ras/MAPK pathway, as occurs in OZR (124). In vitro models further support the notion that selective IR plays an important role in the pathogenesis of vascular dysfunction. For example, simultaneous treatment of endothelial cells with wortmannin

(PI3k inhibitor) and high insulin levels blunts signaling through the PI3k signaling cascade. The lesser PI3k signaling is accompanied by an enhanced signaling through the Ras/MAPK pathway and enhanced mitogenesis, as well as upregulation of vascular cell adhesion molecules VCAM-1 and E-selectin (125) which are known early events in the pathogenesis of vascular dysfunction. Further, in isolated arteries from pigs this phenomenon (persistent insulin signaling with restricted PI3k) has been shown to induce vasoconstriction, rather than vasodilation, and such aberrant response was rescued by MAPK or ET-1 inhibition (126).

In humans there is an augmented ET-1 production in subjects with metabolic syndrome (127) as well as in patients with T2D (128) under resting conditions, and this augmented ET-1 production appears to underlie the impairment in resting endothelial function. In this regard, ETA receptor antagonism increases forearm blood flow to a greater extent in metabolic syndrome patients than control subjects, however, this group difference is abolished during dual ET-1 receptor antagonism (127). In addition, Mather et al. (128) documented a correction in the impairment in methacholine-induced endothelium-dependent vasodilation in both non-diabetic obese and overt T2D subjects following intra-arterial ETA receptor antagonism. Further, in patients with coronary artery disease whom also exhibit T2D both selective ETA and dual ET-1 receptor antagonism similarly improves endothelium-dependent vasodilator responses to serotonin (129). Although, numerically dual ET-1 receptor antagonism was associated with a greater improvement in endothelium-dependent vasodilation than ETA receptor antagonism alone. Another investigation has examined the effect of direct administration of ET-1 on vascular function at rest in IR subjects. In this regard, intra-arterial infusion of ET-1 at rest in older subjects with IR reduces forearm blood flow, and impairs both endothelium- dependent and independent vasodilation (130). Collectively, these data suggest that augmented ET-1 action under resting

conditions contributes to resting endothelial dysfunction in obese, IR, and T2D subjects, and ET-1 receptor antagonism is capable of abrogating this response.

Beyond inducing vascular dysfunction at rest, ET-1 may importantly contribute to impairments in insulin-stimulated vasodilation. Indeed, the blood flow response to hyperinsulinemia, induced pharmacologically or post-prandially, is impaired in both obese IR subjects (92, 131, 132) and patients with T2D (133, 134).

In support that ET-1 directly contributes to these impairments, the expression of ET-1 in skeletal muscle biopsies taken from obese T2D patients was augmented during hyperinsulinemia relative to baseline, while lean insulin sensitive individuals exhibited no such increase in skeletal muscle ET-1 expression (133), which may suggest an insulin-induced upregulation of ET-1 specific to skeletal muscle in these patients. In addition, local ETA receptor antagonism during hyperinsulinemia restores the leg blood flow and leg glucose uptake responses to insulin in obese IR subjects (92). Further, in patients with coronary artery disease whom also display T2D, systemic antagonism of both the ETA and ETB receptors improves whole body insulin sensitivity (135). Contrastingly, systemic ETA receptor antagonism alone appears to be insufficient to improve whole body insulin sensitivity in these patients (135). However, the lack of effect during ETA antagonism in this study may be explained by alterations in the ET-1 pathway specific to coronary artery disease (136), low subject numbers, or perhaps by actions of the ETB receptor in other vascular beds (e.g., kidney) that are accounted for during dual ET-1 receptor antagonism but not during ETA receptor antagonism alone (135, 137).

Interaction of Central and Local Vascular Actions of Insulin in Type 2 Diabetes

Similar to the above sections, the interactions between the central and local vascular actions of insulin in patients with T2D remain largely understudied. Under resting conditions, patients with T2D exhibit augmented vasoconstrictor responses to exogenously administered norepinephrine (123), which could suggest greater α-adrenergic sensitivity in T2D. However, the contribution of chronic hyperinsulinemia to this exaggerated response has not been defined. There is a remarkable paucity of literature regarding the interaction between sympathetic vasoconstriction and the local vascular actions of insulin in T2D. Despite this, the supposed sympatho-attenuating effects of insulin have been studied in essential HTN, and it appears that any putative insulin-induced sympatho-attenuation is absent in HTN (138-140).

Critical Knowledge Gaps and Experimental Hypotheses

Given the above background, it is clear that despite significant advancements, there are still critical knowledge gaps throughout the literature. The most glaring inconsistencies, and thus the topic(s) of this dissertation are as follows: First, it remains uncertain whether or not patients with T2D exhibit augmented sympathetic vasoconstriction, and if indeed this occurs; Does this exaggerated sympathetic vasoconstriction translate into greater changes in BP, thereby contributing to excursions in BP in patients with T2D? In order to adequately address this question, the appropriate model of sympathetic transduction to BP must be identified and then translated to the study of T2D. In Chapter 3, we examined for the first time, sympathetic transduction to BP in patients with T2D compared to age- and body mass index- matched control subjects.

Hypothesis 1: We hypothesized that patients with T2D would exhibit augmented sympathetic transduction to BP compared to age- and body mass index- matched control subjects.

Following positive results in the investigation above, documenting an exaggerated sympathetic transduction to BP in patients with T2D, we sought to determine the role that hyperinsulinemia itself explicitly plays in augmenting sympathetic transduction. Inherently intertwined within this research question is the notion that sympatho-attenuating effects of insulin have been proposed, and equivocal results have been produced, which has ultimately lead to marked speculation as to the net effect of sympathetic vasoconstriction during hyperinsulinemia. Importantly, to date, all of the experimental investigations examining sympathetic vasoconstriction during hyperinsulinemia have hinged upon exogenously administering an α-adrenergic vasoconstrictor or reflexively super-imposing sympathetic vasoconstriction. Therefore, these studies have inherently discounted the fact that insulin naturally stimulates sympathetic outflow. In total then, *net* sympathetic transduction during hyperinsulinemia remains incompletely understood. Therefore, using the same signal-averaging approach, in Chapter 4 we quantified sympathetic transduction at rest and during acute experimental hyperinsulinemia in healthy humans to begin to understand the effects of insulin on sympathetic transduction.

Hypothesis 2: We hypothesized that due to the innate sympatho-excitation inherent to hyperinsulinemia, net sympathetic transduction to BP would remain unchanged during hyperinsulinemia relative to pre-insulin conditions.

Finally, despite substantial animal and preclinical models suggesting an upregulation of ET-1 expression and thus greater ET-1 mediated vasoconstriction during hyperinsulinemia in obesity and IR (92), this has never been investigated in patients with overt T2D. Further, the explicit roles of the ETA and ETB receptor for limiting insulin-stimulated vasodilation remain to be entirely defined. Therefore, in Chapter 4 we mechanistically probe the role that ET-1 plays in

limiting insulin-stimulated vasodilation via local blockade of the ET-1 receptors during hyperinsulinemia in patients with T2D.

Hypothesis 3a: We hypothesized that ETA receptor antagonism would improve insulin-stimulated blood flow to a greater extent in patients with T2D compared to control subjects.

Hypothesis 3b: We hypothesized that dual ET-1 (ETA + ETB) receptor antagonism would further augment this effect.

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Chapter 3

Sympathetic Transduction in Type 2 Diabetes: Impact of Statin Therapy

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Chapter 4

Intact Sympathetic Transduction During Hyperinsulinemia

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INTRODUCTION

The consumption of food substances, particularly carbohydrate, is accompanied by a marked increase in plasma insulin concentration. Beyond the metabolic actions of insulin, post-prandial hyperinsulinemia evokes substantial peripheral vasodilation (1, 2), presumably, in order to facilitate the redistribution of blood flow towards metabolically active tissue (e.g., skeletal muscle). Importantly, the blood flow response to insulin has been shown to contribute ~35% to the glucose uptake in skeletal muscle induced by insulin (2), indicating that insulin-stimulated vasodilation is imperative for glycemic control. However, despite the large peripheral vasodilation, arterial blood pressure (BP) changes only minimally during experimental hyperinsulinemia in healthy humans (3-5), suggesting that this peripheral vasodilation is modulated by counter-acting forces.

It is now firmly established that hyperinsulinemia also elicits a marked increase in sympathetic outflow to the skeletal muscle vasculature [muscle sympathetic nerve activity; MSNA (5-7)], which is predominantly due to the central (i.e., brainstem) rather than peripheral actions of insulin (8). Historically, the increase in MSNA evoked by insulin in the central nervous system has largely been considered as a feedback regulator of the peripheral vasodilator actions of insulin (8-10), although this has recently been called into question (3). An important aspect of insulin signaling, which may contribute to the above noted controversy is the notion that insulin is capable of blunting sympathetic vasoconstriction. The sympatho-attenuating action of insulin signaling has been a topic of much interest, yet previous findings remain equivocal. In vitro experiments generally suggest that insulin is capable of modulating α-adrenergic vasoconstriction (3, 11). Yet in vivo, the vasoconstrictor responses to norepinephrine/α-adrenergic stimuli in the presence of insulin have been found to be attenuated (12-15), unaltered (16, 17) or *perhaps* even augmented

(18). Yet, these studies do not account for the innate sympatho-excitatory features of insulin, so the net effect of insulin on sympathetic vasoconstriction, and thus its influence on sympathetic support of BP remains incompletely understood.

Therefore, in the present investigation we set out to reconcile these purported equivocal findings, in order to further understand BP regulation during hyperinsulinemia. More specifically, the purpose of our study was to examine the beat-to-beat BP and vasoconstrictor responses (sympathetic transduction) to endogenous bursts of MSNA occurring at rest, and during hyperinsulinemia in order to determine if hyperinsulinemia has any functional effect on sympathetic transduction. We hypothesized that due to the innate contrasting local vascular sympatho-attenuating and central sympatho-exciting actions of insulin, the *net* sympathetic transduction to BP would remain unchanged during hyperinsulinemia relative to pre-insulin conditions.

METHODS

Subjects

For the present investigation, five young, healthy men (age: 28 ± 5 yrs; body-mass index: 27 ± 2 kg/m^2) were recruited. This is, at present, on-going work and more experiments will be performed. All subjects provided written informed consent after explanation of the study procedures and experimental measures, which were approved by the Institutional Review Board at the University of Missouri, and conformed with the Declaration of Helsinki. All subjects were free from overt disease, and reported that they did not currently take medication, and did not smoke or use tobacco products.

Experimental Measurements

Heart rate (HR) was measured using a lead II surface electrocardiogram (Quinton; Bothell WA). Beat-to-beat arterial BP was measured via finger photoplethysmography (Finapres Medical Systems; Amsterdam, the Netherlands), and verified periodically by automated sphygmomanometer BP (Welch Allyn; Skanateales Falls, NY) measurements. Respiratory movements were monitored using a strain-gauge pneumobelt secured around the abdomen (Pneumotrace, UFI, Morro Bay, CA) to ensure consistent respiration throughout the protocol. Microneurography was performed to measure post-ganglionic MSNA as previously described (19-21). Briefly, a unipolar tungsten microelectrode was inserted percutaneously into the skin just below the fibular head. The electrode was then positioned into muscle fascicles within the peroneal nerve. The signal was amplified, band-pass filtered (700–2000 Hz), rectified, and integrated (0.1 s time constant) to provide a mean voltage neurogram (Nerve Traffic Analyzer Bioengineering, University of Iowa). MSNA was identified by pulse synchronous bursts and confirmed with muscle afferent stimulation, in the absence of skin afferent stimulation.

Plasma glucose and insulin were measured from venous blood samples drawn from a dorsal hand vein. Glucose was determined using the glucose oxidase method (Thermo Fisher, Waltham, MA, USA or Beckman Instruments, Brea, CA, USA). Insulin was determined using a chemiluminescent enzyme immunoassay (Immulite 1000 Analyzer, Diagnostic Products Corp., Los Angeles, CA, USA). Real-time glucose monitoring (see below) was used (only) to ensure maintenance of euglycemia during insulin infusion, measured via a hand-held glucose meter.

Experimental Protocol

Prior to the study visit, subjects were instructed to arrive to the laboratory at least 12 hours post-prandial, and having refrained from exercise for 48 hours, alcohol for 24 hours, and caffeine for 12 hours. Upon arrival to the laboratory, intravenous catheters were inserted in the left

antecubital, and right dorsal hand vein, for the infusion of insulin/glucose, and for blood sampling, respectively. Then, subjects were instrumented for neural cardiovascular measures. The right hand was placed in a heated box (~50 degrees Celsius) for the arterialization of venous blood, as previously described (22). Insulin (Humulin, Eli Lilly, Indianapolis, IN, USA) was primed for infusion by diluting the insulin in 0.9% isotonic saline combined with 5 mL of the subject's blood. After instrumentation, subjects rested supine for 10-20 minutes in a dimly lit, temperature-controlled room (21-23°C), while HR, BP, respiration, and MSNA were continuously recorded. During this time, baseline (BL) glucose values were determined using the hand-held glucometer. Following these BL measures, a 10-minute infusion of insulin was initiated at a priming dose, which was subsequently followed by constant infusion of insulin at 30 $mU/m^2/min$ for 150 minutes to raise insulin to post-prandial levels. Throughout the insulin infusion, glucose was well-maintained at resting values via a variable 20% dextrose infusion, with the rate dextrose infusion varied (if necessary) based upon arterialized glucose samples obtained every 5-minutes throughout the duration of the clamp, to ensure maintenance of euglycemia. Throughout the entire protocol, subjects were instructed to remain quiet and awake. All neural cardiovascular measures were simultaneously collected at 1,000Hz in commercial acquisition software (PowerLab; AD Instruments) and stored offline for later analysis.

Data Analysis

Resting cardiovascular and neural measures, as well as respiration rate, were calculated as mean values over the duration of the entire resting period before (i.e., at BL), and during insulin infusion. Data for hyperinsulinemia were selected as 20-minute periods around the 120-minute mark based upon visual inspection of the respiration trace to ensure consistent breathing, due to the known influence of respiration on MSNA (23). Mean arterial pressure (MAP) was calculated

as an average of the automated sphygmomanometer BPs obtained over the duration of the resting periods, at BL and during hyperinsulinemia. Stroke volume (SV) was estimated using the Modelflow method (24) and cardiac output (CO) calculated as SV multiplied by HR. Total vascular conductance (TVC) was calculated as CO divided by MAP. Plasma insulin and glucose values represent the average of 2 and 4 plasma sample measurements, respectively, taken within the final 45 minutes of the clamp (in order to correspond with selection of the MSNA data).

Customized LabView (National Instruments, Austin, Texas) software was used to analyze MSNA, as previously described (19, 20, 25). Bursts of sympathetic outflow were identified by pulse synchronicity, signal to noise ratio of 3:1, and morphology of the burst. Neurograms were analyzed for resting MSNA, quantified as burst frequency (bursts/minute) and burst incidence (bursts/100 heartbeats).

The transduction analysis of MSNA into changes in MAP and TVC was performed as previously described (25-27). Briefly, spike-triggered averaging was performed wherein bursts of MSNA acted as triggers and were followed for 10 subsequent cardiac cycles. In this analysis, the change in MAP or TVC is defined as the instantaneous MAP/TVC at each given cardiac cycle subtracted by the MAP or TVC at time point 0. Time-point zero is defined as the cardiac cycle in which the MSNA burst originally occurred. The response over the 10 cardiac cycles for all bursts of MSNA was signal-averaged for each subject. The peak response (MAP or TVC) to bursts of MSNA were chosen within the first 10 cardiac cycles, given peak response latencies are consistently reported within 5-8 heart beats following bursts of MSNA (21, 26, 27).

Statistical Analysis

Normality of all data was assessed using the Shapiro-Wilk test, and when appropriate, nonparametric testing was performed. In the present investigation, non-normally distributed data did not influence the interpretation of any outcome measure. All resting neural cardiovascular and ventilation variables, as well as the peak BP and TVC responses to bursts of MSNA, were compared from baseline (BL) to steady-state hyperinsulinemia using Student's t-tests or Mann-Whitney U test. Beat-to-beat MAP and TVC responses to bursts of MSNA were compared using two-way repeated measures ANOVA with Tukey post-hoc correction. All statistical analysis was completed using commercial statistical software (Sigmastat 13). All data are reported as mean ± SE, and statistical significance was set a-priori at $P<0.05$.

RESULTS

Neural Cardiovascular Responses to the Insulin Clamp

As expected, plasma insulin concentrations were significantly elevated by the infusion of insulin (BL: 34 ± 10 pmol/L; Insulin: 206 ± 33 pmol/L, $p=0.001$), while plasma glucose values were well-maintained during the clamp (BL: 91 ± 2 mg/dL; Insulin: 83 ± 6 mg/dL, $p=0.15$). Neural and cardiovascular variables at BL and during hyperinsulinemia are presented in Table 1.

Figure 1 depicts sympathetic neurograms at BL and during hyperinsulinemia in one representative subject. The induction of hyperinsulinemia significantly augmented MSNA burst frequency and burst incidence (see Table 1). The increase in MSNA burst frequency and incidence was also accompanied by an increase in the average amplitude of the MSNA bursts during hyperinsulinemia (BL: 42 ± 2 Au; Insulin: 59± 3 Au, $p<0.001$).

Sympathetic Transduction During Hyperinsulinemia

The beat-to-beat MAP and TVC responses following spontaneous bursts of MSNA at BL, and in response to hyperinsulinemia are presented in Figure 2. All bursts of MSNA elicited a robust increase in MAP (Fig. 2A) and as well as a robust decrease in TVC (Fig. 2B) over the subsequent 10 cardiac cycles both before, and during hyperinsulinemia (cardiac cycle effects, $P<0.001$). Likewise, the magnitude of the beat-to-beat increase in MAP and decrease in TVC were not significantly different between conditions. The peak increase in MAP (BL: 2.8 ± 0.5 mmHg; Insulin: 2.4 ± 0.3 mmHg, $p=0.42$) and peak reduction in TVC (BL: -3.8 ± 0.4 mL/min/mmHg; Insulin: -3.3 ± 0.9 mL/min/mmHg, $p=0.48$) were also not significantly different between BL and hyperinsulinemia.

Discussion

The major novel finding of the present investigation is that the BP and TVC responses following bursts of MSNA remain unchanged during hyperinsulinemia (i.e., intact sympathetic transduction) in young, healthy men. These data suggest that the contrasting local sympatho-attenuating, and central sympatho-excitatory features of insulin likely converge such that net sympathetic transduction to BP remains unchanged during hyperinsulinemia.

Post-prandial insulin signaling both centrally, and peripherally, contributes importantly to the re-distribution of blood flow towards metabolically active tissue, and thus are requisite for appropriate glycemic control (2, 8). Importantly though, the magnitude of peripheral vasodilation must be tightly controlled in order to ensure the appropriate maintenance of BP. Historically, the role of sympathetic vasoconstriction in tempering insulin-stimulated vasodilation has been well-established (8), although this notion has been recently questioned (3). Our findings might reasonably, in part, reconcile these previous findings. Therefore, it is prudent to discuss the contrasting lines of evidence, and how, together, with the work contained herein, our

understanding of the sympathetic nervous system's contribution to BP regulation during hyperinsulinemia has advanced.

The primary line of work implying attenuated sympathetic restraint of blood flow, and thus reduced sympathetic contribution to BP regulation during hyperinsulinemia stems from the observation in humans that intra-arterial infusion of insulin attenuates α-adrenergic vasoconstriction (14, 15, 28). Although the contributing mechanism(s) for this blunted response to α-adrenergic stimulation remain to be entirely elucidated, there are several factors which may contribute. First, it is plausible that the endothelial-cell dependent signaling pathways activated by insulin directly interact with adrenergic signaling. In this regard, Lembo and colleagues have demonstrated that insulin signaling modulates an endothelial NO component present in both the α_2- and β-adrenergic (14, 28, 29) signaling pathways, and have suggested that these pathways may contribute to the attenuated vasoconstrictor response to intra-arterial norepinephrine that occurs in the presence of insulin (14, 30). Alternatively, this attenuation of sympathetic vasoconstriction by insulin may also include the modulation of α_1- adrenergic signaling (3), although this finding remains equivocal (11, 29). Another possibility is that insulin also modulates norepinephrine kinetics, as in vitro studies have indicated that insulin is capable of attenuating norepinephrine release (31) as well as enhancing the reuptake of norepinephrine (32) by sympathetic nerve terminals, although it does not appear that these effects are obligatory in humans (29). Further, in addition to modulating norepinephrine signaling/kinetics, it is also possible that insulin alters the release of sympathetic co-transmitters (e.g., adenosine triphosphate or neuropeptide Y) at the level of the neurovascular junction. However, measuring alterations in neurotransmitter release at the level of skeletal muscle is technically challenging and to our knowledge has not been completed in humans during hyperinsulinemia. Collectively, these data indicate that insulin could be capable

of 'lysing' sympathetic vasoconstriction, and have led to the suggestion that other mechanisms, namely, increases in cardiac output may be the primary contributor to the maintenance of arterial BP during hyperinsulinemia (3). While plausible, it is important to consider that only modest increases in HR and cardiac output occur during hyperinsulinemia in humans (3-5), and such minor (~300 mL/min) changes in cardiac output (3) would seem insufficient to single-handedly sustain BP in the face of such potent peripheral vasodilation.

On the other hand, several lines of evidence support the notion that peripheral sympathetic vasoconstriction is enhanced, and thus contributes importantly to the maintenance of arterial BP during hyperinsulinemia. Most simple, patients with autonomic failure exhibit dramatic reductions in arterial BP following insulin infusion (33). In addition, intra-arterial phentolamine (non-selective α-adrenergic receptor antagonist) administration during hyperinsulinemia markedly increases forearm blood flow (34), suggesting some level of sympathetic restraint of blood flow persists during hyperinsulinemia. Although, in this investigation, phentolamine infusion was not completed under resting conditions, so the relative change in phentolamine-induced hyperemia (as an index of sympathetic restraint of blood flow) in the presence of insulin remains incompletely understood. Moreover, patients with regional sympathectomy experience insulin-stimulated vasodilation more rapidly in the denervated limb compared to the innervated limb (35). Important to these studies though, the magnitude of vasodilation is greater in the denervated limb only initially, with no difference in the magnitude of peak vasodilation between limbs. This could suggest that sympathetic vasoconstriction plays an especially important role in controlling the kinetics of peripheral vasodilation, rather than the overall magnitude of vasodilation. Collectively, these data suggest that sympathetic vasoconstriction persists during hyperinsulinemia, and the

partial/complete absence of sympathetic vasoconstriction results in more rapid vasodilation, as well as reductions in arterial BP.

While at first glance these two lines of evidence may appear to yield disparate conclusions, our findings might reasonably bridge these two lines of evidence because the studies using arterial infusion of insulin inherently discounted the increase in MSNA that naturally accompanies hyperinsulinemia. Therefore, our data documenting intact BP and TVC responses to spontaneous bursts of MSNA during hyperinsulinemia might reasonably suggest that insulin's central sympatho-excitatory features appropriately offset the peripheral sympatho-attenuating action(s) of insulin. In general agreement with this notion, insulin has been reported to enhance the gain of the arterial baroreflex control of MSNA (5), indicating that insulin's sensitization of the baroreflex control of MSNA may potentiate the capacity of baroreflex-mediated changes in MSNA to regulate arterial BP. Therefore, the interaction between these local and central actions of insulin might simply represent a highly sensitive feed-back regulatory system that cumulatively results in no change in sympathetic transduction, but enables highly sensitive adjustments to blood flow to aid in substrate delivery to metabolic tissues, whilst also maintaining arterial BP.

In summary, we document, for the first time, intact sympathetic transduction to BP during hyperinsulinemia in young, healthy men. Further, given intact sympathetic transduction to BP, and a general increase in MSNA burst frequency/incidence, our data may suggest that sympathetically-mediated vasoconstriction contributes importantly to the maintenance of BP during hyperinsulinemia in young, healthy individuals.

Figures and Tables

Table 1. Neural, Cardiovascular, and Ventilatory Variables at Rest and during Hyperinsulinemia

Parameter	**Baseline**	**Hyperinsulinemia**
SBP (mmHg)	115±3	115±4
DBP (mmHg)	65±1	63±2
MAP (mmHg)	82±1	80±2
HR (beats/min)	59±6	61±5
TVC (mL/min/mmHg)	82.9±5.1	90.8±10.6
Respiration Rate (breaths/min)	12.9±0.5	14.6±0.3*
MSNA Burst Frequency (bursts/min)	14±4	22±3*
MSNA Burst Incidence (bursts/100hb)	25±7	37±6*

Values are mean ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; TVC, total vascular conductance; MSNA, muscle sympathetic nerve activity. BP values presented are the average of automated sphygmomanometer measurements taken throughout the baseline and during hyperinsulinemia. Hyperinsulinemia data represent data from 20-minutes collected ~120-minutes into the insulin clamp. * $P \leq 0.05$ compared to Baseline.

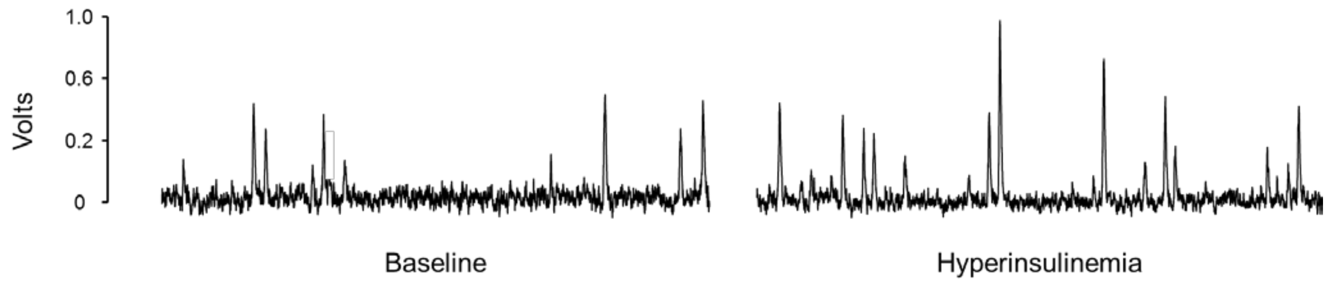

Figure 1. A one-minute representative original recording of muscle sympathetic nerve activity (MSNA) at baseline (left) and during hyperinsulinemia (right) demonstrating marked sympatho-excitation during hyperinsulinemia..

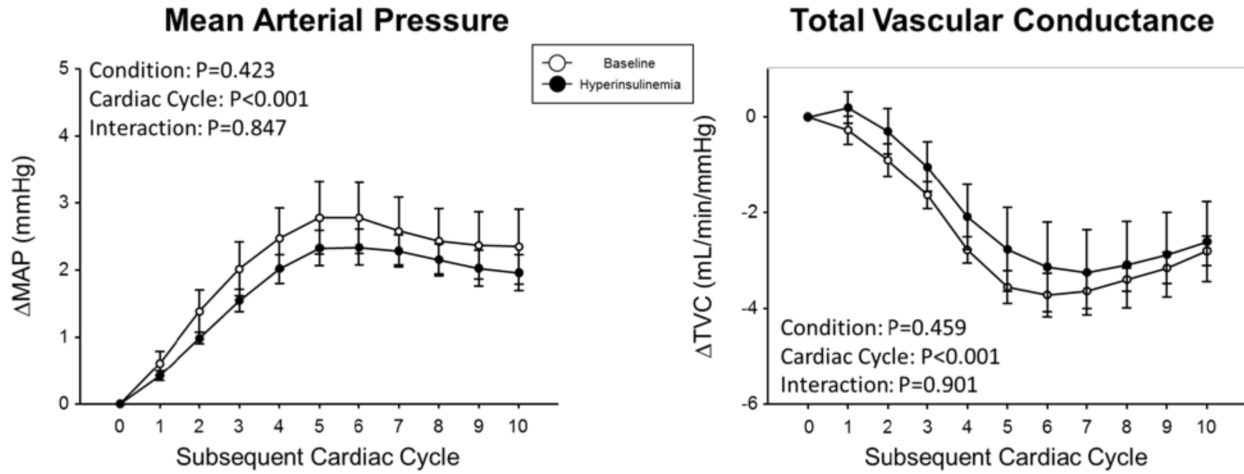

Figure 2. Net sympathetic transduction to blood pressure is not altered during steady-state hyperinsulinemia in young healthy men (n=5). Summary data showing beat-to-beat changes in mean arterial pressure (MAP; left) and total vascular conductance (TVC; right) responses following spontaneous bursts of muscle sympathetic nerve activity (MSNA) at baseline (BL; open circles) and during steady-state hyperinsulinemia (Insulin; closed circles). Data are reported as mean ± SE.

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Chapter 5

Endothelin-1 A Receptor Antagonism Improves Insulin-Stimulated Blood Flow in Patients with Type 2 Diabetes

Benjamin E. Young

INTRODUCTION

Beyond its direct metabolic actions, insulin also stimulates peripheral vasodilation which serves to facilitate glucose delivery to its target tissue (i.e., skeletal muscle) for disposal. Insulin-stimulated vasodilation is achieved through the binding of insulin to its tyrosine kinase receptor on endothelial cells, ultimately activating two distinct signaling cascades with reciprocal actions (1). The first pathway results in phosphatidylinositol 3-kinase - protein kinase B (PI3K-Akt) dependent stimulation of endothelial nitric oxide synthase (eNOS), and the production of nitric oxide (NO). The other pathway is a Ras/mitogen-activated protein kinase (MAPK) dependent signaling cascade that results in the production of endothelin-1 (ET-1). In healthy individuals, the net effect of vascular insulin signaling is a substantial increase in skeletal muscle blood flow (BF), which accounts for ~35% of the skeletal muscle glucose uptake response to insulin (2).

Yet, in patients with T2D insulin-stimulated BF is markedly impaired (3-5), which in turn contributes to glucose intolerance (2, 5, 6). In this regard, data from several lines of animal model investigations suggest that augmented ET-1 production by insulin could underlie the impairment in insulin-stimulated BF in patients with T2D (1). Supporting this notion, data from our laboratory has documented enhanced ET-1 peptide expression in skeletal muscle biopsies taken during hyperinsulinemia in patients with T2D (3). However, augmented ET-1 production in and of itself does not necessarily result in overt vasoconstriction. Rather, the vasoactive effect of ET-1 depends on the sub-population(s) of ET-1 receptors which are stimulated. Indeed, ET-1 binds two distinct receptor subtypes, which have differential actions based on location. When activated, ET-1 A (ETA) receptors located primarily on vascular smooth muscle elicit vasoconstriction. Similarly, activation of vascular smooth muscle ET-1 B (ETB) receptors also produces vasoconstriction. Contrastingly though, activation of endothelial ETB receptors elicits potent nitric-oxide (NO)

mediated vasodilation. In this way, the (Ras/MAPK) ET-1 signal transduction pathway activated by insulin contains its own feed-back regulatory loop, with ET-1 production being capable of eliciting either vasodilation or vasoconstriction.

Several studies have been conducted to better understand the effect of ET-1 on insulin sensitivity. In animals, sustained ET-1 exposure (5-days of continuous micro-infusion using an osmotic minipump) induces both whole-body and skeletal muscle IR (7). Likewise, mice over-expressing ET-1 in endothelial cells also exhibit glucose intolerance (8). These findings are complemented by human studies wherein exogenous ET-1 administration reduces insulin sensitivity, quantified using the hyperinsulinemic-euglycemic clamp methodology (9, 10). Further, studies utilizing ET-1 receptor antagonism have been undertaken in humans with overt IR. Indeed, Lteif et al. (11) demonstrated that local (intra-arterial) administration of an ETA receptor antagonist during hyperinsulinemia was capable of abrogating the impaired insulin-stimulated BF in obese IR subjects, relative to healthy controls. Importantly, rectification of the BF response to insulin also resulted in near-normalization of skeletal muscle glucose uptake in the obese individuals in that investigation. However, these findings do not account for the functional role of the ETB receptor during hyperinsulinemia, nor were they completed in patients with overt T2D. This is important because vascular smooth muscle ETB receptors are upregulated in vitro in the presence of hyperglycemia, and this results in enhanced ETB-receptor mediated vasoconstriction (12), suggesting that T2D could result in a pro-vasoconstrictor phenotype of ETB receptors.

In humans, the role of the ETB receptor during hyperinsulinemia has been investigated in patients with coronary artery disease whom displayed IR. Indeed, Ahlborg et al. (10) documented a significant improvement in whole body glucose uptake during hyperinsulinemia following systemic antagonism of both the ETA and ETB receptor, but no improvement following selective

ETA receptor antagonism in obese patients with coronary artery disease. Yet, systemic administration of these drugs limits the interpretation of mechanism(s), as ET-1 displays actions in other tissues which may impact glucose tolerance, and these other actions may be effected differentially by ETA versus ETB blockade. For example, ET-1 infusion has been reported to alter splanchnic glucose production in man (13), and has also been reported to blunt the acute pancreatic response to glucose (14). Moreover, the endothelin system has been implicated in the pathogenesis of coronary artery disease, and may putatively be differentially impacted by coronary artery disease relative to T2D (15). In total then, the role that ET-1 plays in the skeletal muscle metabolic derangements in patients with overt T2D (i.e., impaired insulin-stimulated BF and glucose uptake), as well as the contributions of the ETA and ETB receptor to these skeletal muscle impairments in T2D have not been comprehensively examined.

With this background in mind, the aim of the current investigation was to quantify insulin-stimulated BF in patients with T2D in the absence, and presence of 1) selective blockade of ETA receptors, and subsequently 2) dual blockade of ETA and ETB receptors. Further, we quantified skeletal muscle glucose uptake (leg balance technique) during these experimental interventions, to determine the extent to which the improvements in insulin-stimulated BF translate into improvements in glucose disposal. We tested the hypothesis that ETA receptor blockade would improve insulin-stimulated BF to a greater extent in patients with T2D compared to control subjects. In addition, given that vascular smooth muscle ETB receptors are upregulated in vitro in the presence of hyperglycemia (12), we also hypothesized that dual ET-1 receptor antagonism would further augment this effect. Finally, we hypothesized that improvements in insulin-stimulated BF would proportionately improve glucose disposal.

METHODS

Ethical Approval and Participants

The experimental measurements and study procedures were approved by the Ethics Committee of the Capital Region, and conformed to the Declaration of Helsinki. Accordingly, this investigation was registered at clinicaltrials.gov. Participants were recruited via advertisements in local newspapers, or from the Odense University Hospital patient database (patients with T2D only). Prior to initiation of the experiment, all subjects provided written informed consent after explanation of the study procedures and experimental measures. All patients with T2D had a medical diagnosis of T2D. Exclusion criteria for the present investigation included: actively having or being treated for known diabetic retinopathy, nephropathy, or neuropathy, ischemic/non-ischemic heart disease, unstable angina, chronic kidney disease, intermittent claudication. Further, subjects were excluded if they had undergone surgery within the last 6 months, or reported smoking or excessive alcohol intake. In total, 22 individuals (10 healthy and 12 patients with T2D) visited the laboratory. Of the patients with T2D, data from 3 patients with T2D are excluded on the basis of inadequate data collection due to 1) urgent need to use restroom during the insulin clamp (n=1) resulting in loss of steady-state hyperinsulinemia; 2) inability to clamp plasma glucose (n=1); 3) complaint of back-pain during the procedure resulting in restlessness and loss of ultrasound data (n=1). Therefore, data are presented for 9 patients with T2D and 10 control subjects. All 19 subjects completed the protocol up to the point of ETA receptor antagonism. However, due to technical challenges associated with the nature of the invasion and duration of experiments, the final stage of the study (ETA + ETB receptor antagonism) was only completed in 14/19 subjects (n=7 in each group). Of the patients with T2D, medication prescription included statin therapy (n=7), ACE-inhibitors (n=3), thiazides (n=3), angiotensin II receptor blocker (n=1), and calcium channel blockers (n=2). In order to control for the effect of anti-hypertensive treatment

on the experimental outcomes, two control subjects taking anti-hypertensive medication were also recruited and both currently reporting taking each of the following: a thiazide, ACE inhibitor, and statin.

Experimental Protocol

This investigation was conducted in accordance with recently-published guidelines for assessing resistance vessel function in human skeletal muscle (16). Prior to the experimental day, subjects were instructed to refrain from alcohol, caffeine, exercise, and glucose-lowering medications for 24 hours. All studies were conducted following an overnight fast. Upon arrival to the laboratory, height and weight were measured and subjects then laid semi-recumbent in a dimly-lit temperature-controlled room (21-24 °C) and catheterization was initiated.

Four catheters (20GA, Arrow International Inc., Reading, PA, USA) were placed in the present study in each individual. All femoral catheters were inserted at a level proximal to the bifurcation of the common femoral artery using the Seldinger technique, under local anesthesia (Xylocain, Astra Zeneca, Mölndal, Sweden) (17). In the experimental (right) leg, a catheter was inserted into the femoral artery and advanced 10cm in the proximal direction, for the infusion of ET-1 receptor antagonists. In addition, in the same leg, a catheter was inserted into the femoral vein and advanced 10cm proximally for venous blood sampling. In the contralateral leg, another femoral arterial catheter was inserted in similar fashion, for blood pressure monitoring and arterial blood sampling. Finally, in one arm, a venous catheter was inserted into the antecubital vein for the infusion of insulin and glucose. On two occasions, catheterization of one of the femoral arteries failed. In these instances, the experiment was continued, and arterial blood pressure (BP) was measured from the same catheter administering the ET-1 receptor antagonists. During the BP

measurements, ET-1 receptor antagonist infusion was briefly halted (~30 seconds) to allow for accurate arterial pressure assessment.

The experimental timeline is detailed in Figure 1. Following catheterization, subjects were allowed to rest quietly for ~30 minutes. During this time, blood was collected and sent to the university hospital for a basic metabolic and lipid panel. Then, baseline (BL) measurements were completed over a 20-minute resting period. Once these BL measurements were completed, subjects underwent a hyperinsulinemic-euglycemic clamp in which plasma insulin was elevated to postprandial concentrations, as originally described by Defronzo (18). Briefly, Insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) was diluted in 0.9% saline with 5 mL of the subject's blood, and infused intravenously into the antecubital vein for 10 minutes at a priming dose. Following this priming dose, insulin was infused at a constant rate of 40 $mU/m^2/min$. Arterial blood glucose was determined every 5 minutes throughout the duration of the protocol using a commercial blood gas analyzer (ABL800 FLEX, Radiometer, Brønshøj, Denmark) and these measurements were used to vary the glucose infusion (20% dextrose) rate to ensure maintenance of euglycemia. Following ~60-180 minutes, based on individual insulin sensitivity, steady-state hyperinsulinemic-euglycemia was achieved defined as three consecutive arterial blood glucose samples within ± 0.2 mmol/L of each other, and requiring little to no (<5%) change in glucose infusion rate. Once steady-state was established, data was collected over a 30-minute period to establish the response to insulin alone. Upon conclusion of insulin alone measurements, the ETA receptor antagonist BQ-123 was infused intra-arterially (25nmol/min) until steady-state euglycemia was re-established, or for up to one hour. Then similarly, the ETB receptor antagonist BQ-788 (25nmol/min) was co-infused with BQ-123 for up to an additional hour. During the intra-

arterial infusions, glucose infusion rates were changed throughout (if need be) to account for any changes in glucose uptake mediated by these drugs.

Over the course of the final 20-minutes of baseline, and during each stage of the insulin clamp (insulin alone, insulin + BQ-123, insulin + BQ-123 + BQ-788) leg BF and arterial BP measurements, as well as arterial and venous blood samples were taken in triplicate (each measure separated by ~10-minutes). Common femoral artery diameter and blood velocity were measured in the experimental leg using duplex Doppler ultrasound (Logic e9, GE Medical Systems, Milwaukee, MI) as previously described by our group (17, 19). All Doppler ultrasound measurements (at minimum 30 seconds in duration) were taken to immediately coincide with each blood sample and stored on the ultrasound in raw form for later analysis. Arterial BP was measured continuously throughout the duration of the protocol at 1,000 Hz on a commercial acquisition platform (Powerlab; AD Instruments) and stored for later analysis. Arterial and venous blood samples were centrifuged to isolate plasma and stored for later analysis of glucose, insulin, and ET-1. Glucose values reported are obtained from the commercial blood gas analyzer because preliminary data from our group suggests these values track well with those measured via the glucose oxidase method. Insulin was measured using an insulin enzyme-linked immunosorbent (ELISA; Mercodia AB, Uppsala, Sweden) assay. ET-1 was measured using a chemiluminescent immunoassay (QuantiGlo ELISA kit, R&D Systems, Minneapolis, Minnesota).

Data Analysis

Approximately 2-3 minutes of beat-to-beat data, as well as ~30 second video files of common femoral artery blood velocity and diameter were collected around each blood sampling time-point. These values were then averaged with respect to the other blood-sampling time-points (see above) within each respective stage of the clamp. R-R interval was calculated from the arterial

blood pressure waveform as the time between systoles. Heart rate was calculated as the inverse of R-R interval. Mean arterial pressure (MAP) was calculated as 1/3* systolic blood pressure + 2/3* diastolic blood pressure. Common femoral artery blood velocity and diameter were analyzed directly on the Doppler ultrasound. Briefly, time-averaged, intensity-weighted mean blood velocity (V_{mean}) was calculated in 5-beat averages, coinciding with an arterial diameter measurement. Leg BF (LBF) was calculated as $V_{mean} \times \pi \times (\text{arterial diameter}/2)^2 \times 60$. Leg vascular conductance (LVC) was calculated as LBF/MAP.

Blood samples were assayed (in duplicate) from two separate time-points within each stage of the protocol and the values averaged to determine arterial and venous concentrations of glucose, insulin, and ET-1. In the event of a high coefficient of variation between duplicate wells, the value for the other time-point within that stage was used alone. Arterio-venous (A-V) difference of glucose, insulin, and ET-1 were calculated as the arterial concentration minus the venous concentration. Leg glucose uptake (LGU) was calculated as A-V glucose difference x LBF. Skeletal muscle clearance of insulin was measured as the extraction ratio x plasma flow [LBF x (1-hematocrit)] as described previously (20). ET-1 secretion was measured, as the A-V difference of ET-1 x plasma flow.

Statistical Analysis

All data are presented as mean ± SD. The statistical analysis was conducted using the commercial statistical package Sigmaplot 13 (Systat Software; unpaired t tests and 1-way repeated measures ANOVA, linear regressions) or SPSS version 24 (mixed model ANOVAs only). Normality was assessed using the Shapiro-Wilk test, and when appropriate, nonparametric testing was performed. Baseline subject comparisons between groups were made using unpaired t-tests. All cardiovascular and hemodynamic, as well as metabolic data were analyzed to compare group

and insulin clamp stage using mixed model ANOVA with Tukey post-hoc correction. However, when these models did not allow for within or between group comparisons of the stages of the clamp (i.e., lack of an interaction P-value), the response in each group was defined using 1-way repeated measures ANOVA. Then, the group comparison was made using an unpaired t-test for the effect of each insulin clamp stage alone (e.g., Figure 2 panel C and D). In addition, to better understand the impact of an individual's insulin sensitivity (i.e., glucose infusion rate) on the resulting responsiveness to BQ-123 or BQ-123 + BQ-788, linear regression analysis was performed (independent variable: glucose infusion rate at end steady-state hyperinsulinemia). Significance was set a priori at a $P<0.05$.

RESULTS

Subject Characteristics and Resting Hemodynamics

Patients with T2D and control subjects were well matched for age (T2D: 56 ± 7 years, control: 51 ± 7 years, P=0.1) and body-mass index (T2D: 27 ± 2 kg/m^2, control: 25 ± 3 kg/m^2; P=0.25). Fasting metabolic parameters for both groups are provided in Table 1. As expected, the patients with T2D had a significantly greater fasting blood glucose, HOMA-IR, and glycated hemoglobin (HbA1c) compared to control subjects. Resting systolic BP (CON: 123 ± 10 mmHg; T2D: 143 ± 7 mmHg, P=0.02), and MAP (CON: 95 ± 4 mmHg; T2D: 106 ± 6 mmHg, P=0.03) were significantly greater in patients with T2D compared to control subjects, although diastolic BP (CON: 81 ± 2 mmHg; T2D: 87 ± 6 mmHg, P=0.13) and heart rate (CON: 71 ± 16 beats/min; T2D: 65 ± 10 beats/min, P=0.49) were similar between groups. Resting LBF (CON: 369 ± 185 mL/min; T2D: 243 ± 105 mL/min, P=0.09), LVC (CON: 3.9 ± 2.2 mL/min/mmHg; T2D: 2.5 ± 1.3 mL/min/mmHg, P=0.15) and LGU (CON: 0.14 ± 0.09 mmol/min; T2D: 0.13 ± 0.12 mmol/min, P=0.85) were not significantly different between groups.

Plasma insulin and glucose concentrations at baseline, and throughout the clamp are provided in Figure 2 (panel A and B), demonstrating maintenance of hyperinsulinemic-euglycemia throughout the protocol.

Effect of ET-1 A receptor antagonism on insulin-stimulated BF and glucose uptake

Insulin clearance by skeletal muscle significantly increased across stages of the clamp (stage, P<0.001) without a difference between groups (Group, P=0.473). Hyperinsulinemia increased skeletal muscle insulin clearance in both patients with T2D (overall P<0.001) and control subjects (overall P<0.001), with no further increase observed during BQ-123 infusion in either group. In addition, there was no difference in plasma venous or arterial ET-1 concentrations between the groups at baseline or throughout the protocol (data not shown). There was a slight but significant reduction in MAP across the stages of the protocol, but no difference between groups (CON, BL: 96 ± 12 mmHg; Insulin: 91 ± 12 mmHg; BQ-123: 87 ± 12 mmHg; T2D, BL: 105 ± 18 mmHg; Insulin: 101 ± 20 mmHg; BQ-123: 94 ± 22 mmHg, Interaction P=0.679). Heart rate did not change significantly throughout the stages of the clamp or in either group (Interaction P=0.34). Glucose infusion rate at the end of insulin alone, as an index of insulin sensitivity was reduced in patients with T2D compared to control subjects, however, this did not reach statistical significance (CON: 5.0 ± 1.7 mg/kg/min; T2D: 3.5 ± 1.6 mg/kg/min, P=0.10).

Figure 3 displays the absolute leg BF (panel A) and leg glucose uptake (panel B) values at BL, during insulin alone, and during insulin + BQ-123 in both the control subjects and patients with T2D. Both groups exhibited an increase in LBF across the stages of the clamp (Stage, P<0.001; see Figure 3). Interestingly, LBF was different throughout the protocol between groups (Group, P=0.022), but not at any specific time-point (Interaction, P=0.106). Likewise, both groups exhibited an increase in LGU in response to insulin as well as in response to insulin + BQ-123

(Interaction P<0.001, all within condition P-values <0.05) but there was no difference in LGU at any stage of the clamp between groups. When the changes in LBF and LGU from BL to insulin alone and insulin + BQ-123 were compared between groups, there was no significant difference between groups (see Figure 3 panel C and D). However, within group, the patients with T2D did not exhibit an increase in LBF in response to insulin alone (P=0.10), while insulin + BQ-123 resulted in a significant increase in LBF that was greater than both resting LBF and LBF during hyperinsulinemia (Overall P<0.001). In contrast, the control subjects experienced a significant increase in LBF in response to insulin alone, which was not further augmented by insulin + BQ-123 (P=0.41). When the LBF responses to insulin alone were compared between groups, the patients with T2D exhibited a significantly blunted increase in LBF compared to control subjects. (CON: 181 ± 156 mL/min; T2D: 58 ± 43 mL/min, P=0.04). Despite not reaching statistical significance the percent change in LBF in response to insulin alone was also substantially reduced (CON: 82 ± 30%; T2D: 29 ± 13%, P=0.12) in the patients with T2D. Likewise, the absolute value change in LVC (P=0.04) in response to insulin was significantly blunted in the patients with T2D, and the percent change in LVC (CON: 84 ± 35%; T2D: 40 ± 14 %, P=0.22) was substantially reduced in T2D compared to the control subjects. The administration of BQ-123 during hyperinsulinemia resulted in nearly twice the increase in LBF and LVC in the patients with T2D relative to the control subjects (e.g., LBF; CON: 66 ± 94 mL/min; T2D: 111 ± 82 mL/min, P=0.22). Therefore, the patients with T2D exhibited a significantly greater percent increase in LBF (Figure 4, panel A), as well as a significantly greater percent increase in LVC (CON: 22 ± 25%; T2D: 49 ± 19 %, P=0.03) compared to the control subjects. In contrast, examination of the change in LGU induced by BQ-123 in both groups (Figure 4, panel B) indicated that BQ-123 increased LGU to a similar extent in both groups.

Effect of Dual ET-1 receptor antagonism on insulin-stimulated BF and glucose uptake.

The co-infusion of BQ-788 along with BQ-123 (i.e., insulin + BQ-123 + BQ-788) did not significantly change insulin clearance by skeletal muscle, MAP or HR (e.g., MAP; CON, Insulin + BQ-123: 85 ± 13 mmHg; Insulin + BQ-123 + BQ-788: 84 ± 12 mmHg; T2D, Insulin + BQ-123: 100 ± 18 mmHg; Insulin + BQ-123 + BQ-788: 101 ± 19 mmHg, P=0.419) in either group. Figure 5 displays the LBF (panel A) and LGU (panel B) throughout each stage of the clamp in the control subjects and patients with T2D whom underwent BQ-788 infusion, while panel C and panel D represent the change in LBF (C) and LGU (D) relative to baseline BF at each stage of the clamp. In this cohort of subjects, similar conclusions were drawn for the insulin alone and insulin + BQ-123 as noted above (data not shown). Infusion of BQ-788 did not cause a significant increase in LBF in either group (both P>0.05). Moreover, both the absolute value change (P=0.33), and percent change in LBF in response to the addition of BQ-788 (Figure 6, panel A) were not significantly different between groups. Analysis of LVC responses to BQ-788 yielded similar results (data not shown). Likewise, co-infusion of BQ-788 did not significantly change LGU in either group (Figure 6, panel B). Interestingly, co-infusion of BQ-123 + BQ-788 resulted in a significant increase in skeletal muscle ET-1 secretion compared to insulin + BQ-123, although this was not significantly different between groups (Interaction P=0.77).

Relationship Between Insulin Sensitivity and Responsiveness to ETA or ETA + ETB blockade

There was no relationship between glucose infusion rate and the LBF percent change in response to BQ-123 administration in the cohort of subjects (R^2=0.01, P=0.673). Further, there was no relationship between glucose infusion rate and the change in LGU in response to BQ-123 (R^2=0.188, P=0.107). Likewise, there was no relationship between glucose infusion rate and the

LBF or LGU response to insulin + BQ-123 + BQ-788, when compared to the response to insulin alone or insulin + BQ-123 (data not shown).

DISCUSSION

The primary novel findings of the present investigation are three-fold: (1) ETA receptor antagonism significantly improves insulin-stimulated BF in patients with T2D, with the percent change in LBF greater in patients with T2D compared to control subjects; (2) superimposing ETB receptor antagonism on top of ETA receptor antagonism during hyperinsulinemia had no additive beneficial effect on insulin-stimulated BF (3) Despite the augmented LBF response to BQ-123 administration in the patients with T2D, the increase in LGU associated with BQ-123 administration was similar between patients with T2D and control subjects. Taken together, these findings suggest that the ETA receptor functionally limits insulin-stimulated BF in patients with T2D, however, the ETB receptor does not appear to play a major (additional) role in limiting insulin's vasodilatory actions in patients with T2D. Further, despite augmenting insulin-stimulated BF to a greater extent in the patients with T2D, both groups exhibited significant increases in LGU in response to ETA receptor antagonism, which were similar between groups.

In the present study, we documented a markedly impaired LBF response to insulin in patients with T2D, which generally agrees with previous work from our group (3, 5), as well as from others (4). While sub- "statistically significant" differences in baseline BF between groups hindered direct (group*stage) comparisons, insulin-stimulated BF was ~50% lower in the patients with T2D compared to control subjects, and BQ-123 induced an ~25% greater increase in BF in the patients with T2D. Taken together, ETA receptor antagonism was only capable of restoring approximately half of the BF response to insulin in the patients with T2D. One primary explanation for this finding may be a continued restraint of insulin-stimulated production of NO, despite ET-1

antagonism. While plausible, the insulin receptor signaling pathways which produce NO and ET-1 appear to be reciprocal, such that antagonism of either arm of insulin signaling enhances the action of the other pathway (11, 21-23). Supporting the notion that ET-1 antagonism results in enhanced insulin-stimulated NO production, BQ-123 administration in obese IR individuals nullifies the original difference in the vasoconstrictor response to NO synthase blockade relative to control subjects (24), indicating that ETA receptor antagonism abolished the impairment in NO production/bioavailability. Unfortunately, the addition of NO synthase blockade was not feasible in the present study, as it would require separate trials (3 study days) so the NO synthase blockade could be completed in the presence of insulin alone, insulin + BQ-123, and insulin + BQ-123 + BQ-788 while not effecting the results of the sequential clamp protocol employed herein. We have also elected not to include measures of NOx flux, on the basis that these measures become highly variable during BQ-123 administration (11), and may be viewed as erroneous under conditions of high flow. Therefore, while we are unable to definitively rule out impaired insulin-stimulated NO production as the primary defect, we believe discussion of other mechanisms beyond the PI3k-Akt and Ras/MAPK pathways activated by vascular insulin signaling is warranted, as they might plausibly contribute to the remaining impairment in insulin-stimulated BF in T2D.

In this regard, impaired insulin-stimulated BF in T2D might reasonably also be related to structural abnormalities along the vascular tree (e.g., microvascular rarefraction) (25) or endothelial dysfunction that occurs secondary to renin-angotensin system activation, greater oxidative stress, the presence of advanced glycation end products or enhanced flux through the hexosamine biosynthetic pathway (1). In this regard, structural abnormalities may be an appealing hypothesis because the maximal LBF response to very high doses of insulin (i.e., maximal insulin stimulation) remains blunted in patients with T2D, whereas maximal insulin-stimulated BF

appears similar between control subjects and obese individuals (4). Beyond those mechanisms, enhanced sympathetic vasoconstriction may also contribute to the impaired insulin-stimulated BF in patients with T2D. Indeed, in health, an overlooked action of vascular insulin signaling is that it is capable of attenuating reflexively-induced sympathetic vasoconstriction (26), indicating partial 'lysis' of sympathetic vasoconstriction during hyperinsulinemia. Importantly, this sympatho-attenuating effect of local vascular insulin signaling is absent in conditions that are highly co-morbid with T2D [e.g., HTN (27)], as well as in isolated arteries from T2D mice (28). In addition, our laboratory has recently produced data documenting augmented BP and total vascular conductance responses to spontaneous bursts of MSNA at rest in some patients with T2D (i.e., sympathetic transduction), further implying sympathetic vasoconstrictor responsiveness is enhanced in T2D (29), and that exaggerated sympathetic vasoconstriction might reasonably play a role in limiting insulin-stimulated BF in these patients.

Consistent with the improvement in insulin-stimulated BF, we also documented an increase in LGU during ETA receptor antagonism in patients with T2D. Although, in this study the increase in LGU was not significantly different between the patients with T2D and the control subjects. These data are in stark contrast to previous work in obese IR subjects, wherein rectifying the BF response to insulin resulted in complete abolishment of the impairment in skeletal muscle glucose uptake in those participants (11). We believe there are multiple considerations that may contribute to the explanation of this finding. First, the most plausible explanation is that despite a greater improvement in LBF in the patients with T2D, ET-1 receptor antagonism failed to correct the metabolic derangements (e.g., impaired GLUT-4 translocation) associated with peripheral vascular IR. If true, a given amount of LBF may result in only a fraction of the improvement in glycemic control in a patient with T2D relative to a healthy, insulin-sensitive individual. We favor

this hypothesis, based on the following logic: There is precedent to suggest that our control subjects would exhibit some level of ET-1 tone since our control subjects were significantly older, and some exhibited less than optimal resting BP (to match for age and anti-hypertensive regimen of the patients). Indeed, ET-1 is known to increase across the age and BP continuums, (30-32), so it may not be surprising that some of our controls exhibited improvements in LBF following ET-1 receptor antagonism. Yet, since our control subjects did not exhibit overt metabolic impairments to insulin administration (evidenced by relatively normal insulin sensitivities), the lesser increase in LBF may have been sufficient enough to induce proportionately greater skeletal muscle glucose uptake, resulting in the observation of similar increases in LGU between groups.

Theoretically, this may also explain the differences between the observed rectification of LGU responsiveness in obese IR subjects during ETA antagonism documented previously (11) compared to the T2D subjects herein. To be explicit, the previous study used an insulin infusion dose that would be considered supra-physiological (i.e., 300 $mU/m^2/min$). That dose is ~7.5 fold higher than the dose administered herein. Therefore, it is conceivable that at such high levels of insulin infusion, the impairment(s) in the metabolic actions of insulin were minimized in the obese IR subjects. Simply, maximal insulin stimulation may have been sufficient enough to induce maximal GLUT-4 translocation in both healthy controls and the obese IR subjects, thereby magnifying the effect of impairments in BF on the impairment in LGU (i.e., during maximal insulin stimulation BF might reasonably be the limiting reagent). In this regard, recent invasive human work has demonstrated that the BF response to insulin acts as an important mediator of interstitial glucose concentration, and thus contributes to the ability of glucose uptake in skeletal muscle, when the membrane permeability to glucose is high (33). Supra-physiological insulin administration might reasonably elevate membrane permeability to glucose beyond that which

occurs under normal physiologic/post-prandial levels, thereby magnifying the influence of defective insulin-stimulated vasodilation on skeletal muscle glucose uptake. Therefore, in our investigation using sub-maximal insulin stimulation, metabolic derangement may have persisted in the patients with T2D, such that improving glucose delivery (i.e., LBF) provided some, but not the same level of improvement in glucose uptake relative to control subjects *per unit increase in BF*.

We would also like to acknowledge that in vitro studies demonstrate that ET-1 signaling in skeletal muscle directly impairs glucose uptake, independent of BF (34-36). Therefore, it is plausible that mechanisms beyond ET-1 contribute to the impairment in the metabolic component to insulin signaling, which are unique to T2D relative to its precursor(s). Indeed, impairments in insulin-stimulated BF due to elevated ET-1, and its associated reduction in skeletal muscle glucose uptake (either directly, or indirectly) might reasonably occur prior to the development of overt impairments in the metabolic actions of insulin.

PERSPECTIVES

Our findings clearly demonstrate that ETA receptor antagonism is capable of (partially) restoring the vasodilator action of insulin in patients with T2D. Further, the improvement in insulin-stimulated BF is indeed associated with an increase in LGU. This is important, because the magnitude of prevailing hyperglycemia is positively related to cardiovascular risk (37), so any improvement in LGU in a population with impaired glucose tolerance (independent of the response relative to controls) may confer cardiovascular protection. Therefore, clinically, ET-1 receptor antagonism may represent a therapeutic intervention for the treatment of skeletal muscle IR in patients with T2D. Indeed, our study was designed specifically to mirror therapeutic potential, as both selective ETA receptor antagonists (e.g., ambrisentan) and non-selective ET-1 receptor

antagonists (e.g., bosentan) are currently FDA-approved and clinically available. In this regard, our data provide the first evidence that concurrent ETA + ETB receptor antagonism provides little further benefit relative to selective ETA antagonism for skeletal muscle glucose uptake in patients with T2D.

Among its diverse actions, ET-1 plays an important role in regulating splanchnic glucose production (13), may alter the acute pancreatic response to insulin (14), and also has well-documented actions in the kidney (38). Thus, it may be suggested that the choice between selective ETA versus non-selective ET-1 receptor antagonists might be informed based upon the therapeutic potential of antagonizing these receptor isoforms outside of skeletal muscle. For example, in patients with coronary artery disease systemic non-selective ET-1 receptor antagonism was superior to selective ETA antagonism for whole body insulin-sensitivity (10). Finally, our data demonstrating an ability of ETA receptor antagonism to improve insulin-stimulated BF in patients with T2D might reasonability suggest that ET-1 blockers be combined with therapeutics to target the impairment in the metabolic actions of insulin, or exogenous insulin administration to synergistically improve glucose uptake. Whether or not combination of ET-1 receptor antagonism and insulin-sensitizing agents elicits an additive (i.e., combined) effect on skeletal muscle glucose uptake remains to be defined.

In summary, we document for the first time, an improvement in insulin-stimulated BF in patients with T2D following ET-1 receptor antagonism. Further, LGU was enhanced in patients with T2D following ET-1 receptor antagonism. Finally, super-imposing ETB receptor blockade on top of ETA receptor antagonism had little further beneficial effect on insulin-stimulated BF, or LGU in patients with T2D.

Figures and Tables

Table 1: Baseline Metabolic Measurements

	CON	T2D	P Value
Glucose (mmol/L)	5.3 ± 0.4	6.6 ± 1.1*	0.008
Insulin (µIU/mL)	5.1 ± 2.8	8.5 ± 6.1	0.15
HOMA-IR	1.0 ± 0.6	2.5 ± 1.5*	0.048
LDL (mmol/L)	2.5 ± 0.7	1.9 ± 0.6	0.23
HDL (mmol/L)	2.1 ± 0.6	1.2 ± 0.4*	0.02
HbA1c (mmol/mol)	32 ± 2	51 ± 7*	<0.001
Triglyceride (mg/dL)	1.1 ± 0.7	1.9 ± 0.7	0.14
Endothelin-1 (pg/mL)	0.77 ± 0.22	0.74 ± 0.28	0.89

Values are means ± SD; CON, control subjects (n=10); T2D, patients with type 2 diabetes (n=9); HOMA-IR, Homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HbA1c, glycated hemoglobin. * P<0.05 patients with T2D compared to CON.

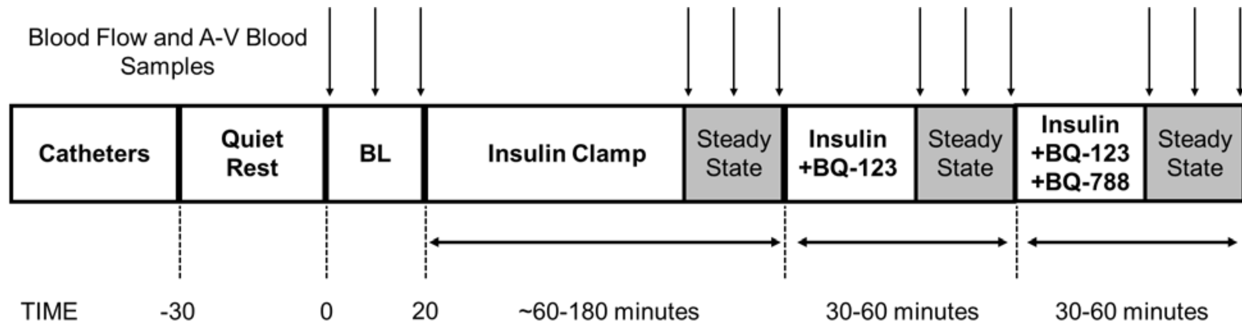
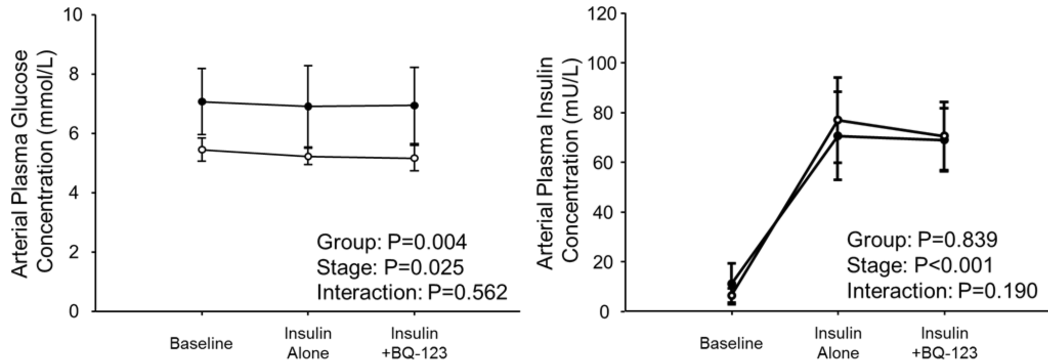


Figure 1. Schematic of the experimental protocol, detailing time-points at which measurements were taken. Subjects underwent catheterization, and were then allowed to rest quietly for ~30 minutes. Following this resting period, baseline measurements were taken in triplicate over 20-minutes. Subjects then underwent a hyperinsulinemic-euglycemic clamp, and following establishment of steady-state hyperinsulinemia (see methods), BQ-123 (endothelin-1; ET-1 A receptor antagonist; 25nmol/min) was infused intra-arterially in the femoral artery until steady-state hyperinsulinemia-euglycemia was re-established. In a subset of participants, following BQ-123, BQ-788 (ET-1 B receptor antagonist; 25 nmol/min) was co-infused with BQ-123, similarly.

A



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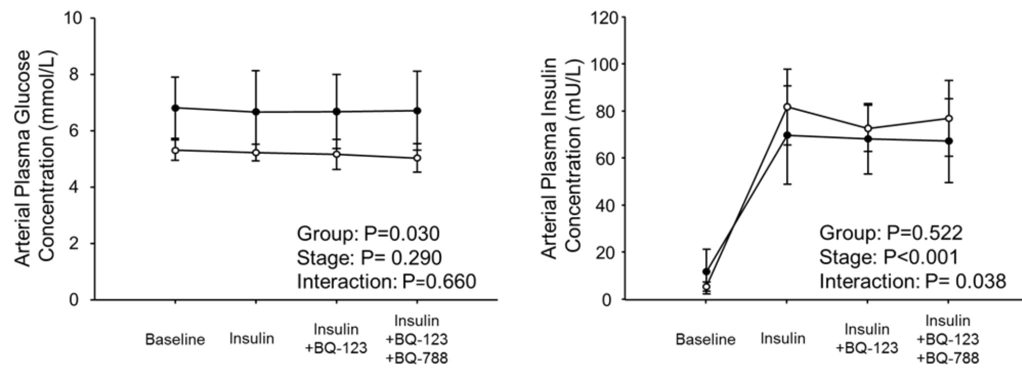

Figure 2. Panel A demonstrates successful maintenance of hyperinsulinemic-euglycemia throughout the protocol in all control subjects and patients with type 2 diabetes (T2D) whom completed BQ-123 infusion (n=10 and n=9, respecitvely). Panel B similarly demonstrated hyperinsulinemic-euglycemia in the subset of participants whom completed BQ-123 as well as BQ-123 + BQ-788 (Panel B, n=7 and n=7, respectively).

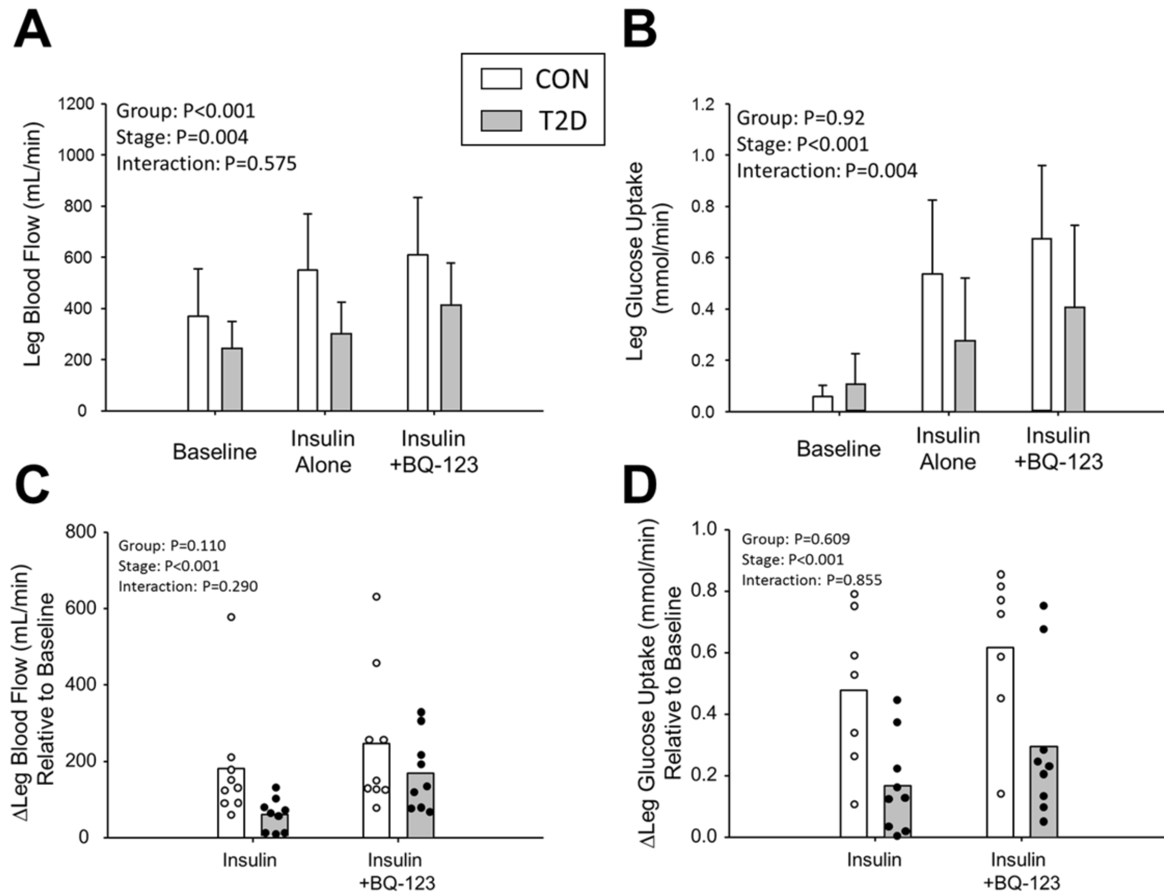

Figure 3. Panels A and B: Summary data for patients with Type 2 Diabetes (T2D; n=9) and control subjects (CON; n=10) showing absolute value leg blood flow (panel A) and leg glucose uptake (panel B) at baseline, during the insulin clamp (insulin alone), and during the insulin clamp with intra-arterially infused Endothelin-1 A receptor antagonist BQ-123 (insulin + BQ-123). Panel C depicts the change in leg blood flow in response to insulin alone and insulin+ BQ-123 administration relative to baseline in both groups. Panel D depicts the increase in leg glucose uptake in both groups in response to insulin alone and insulin + BQ-123 relative to baseline.

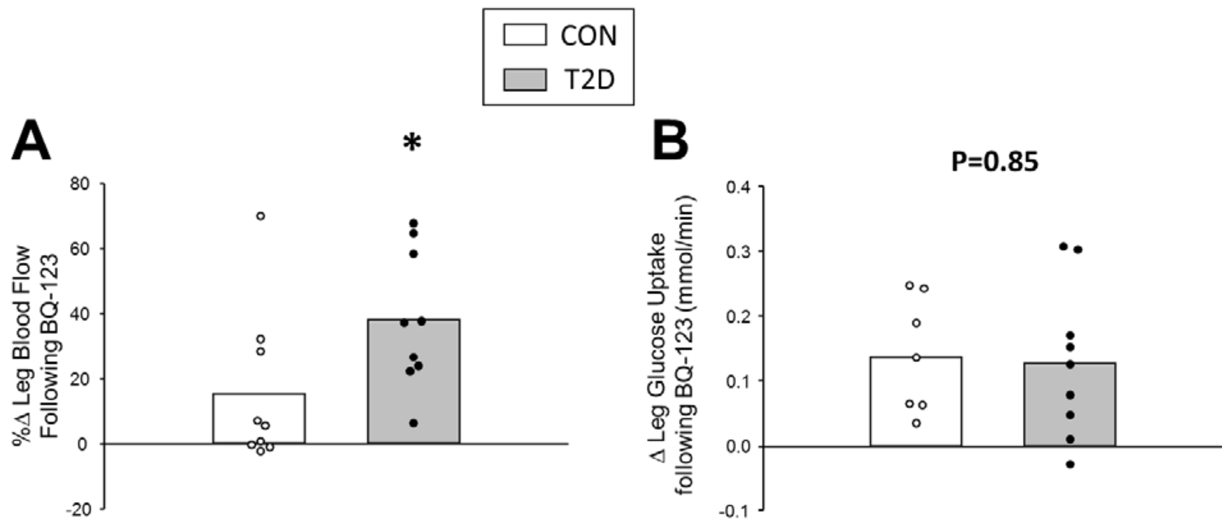

Figure 4. Panel A demonstrates a significant percent increase in response to BQ-123 (insulin alone versus insulin + BQ-123) in the patients with Type 2 Diabetes (T2D) compared to control (CON) subjects. Panel B demonstrates the change in leg glucose uptake, similarly, which was not different between groups. * P<0.05 patients with T2D vs. CON.

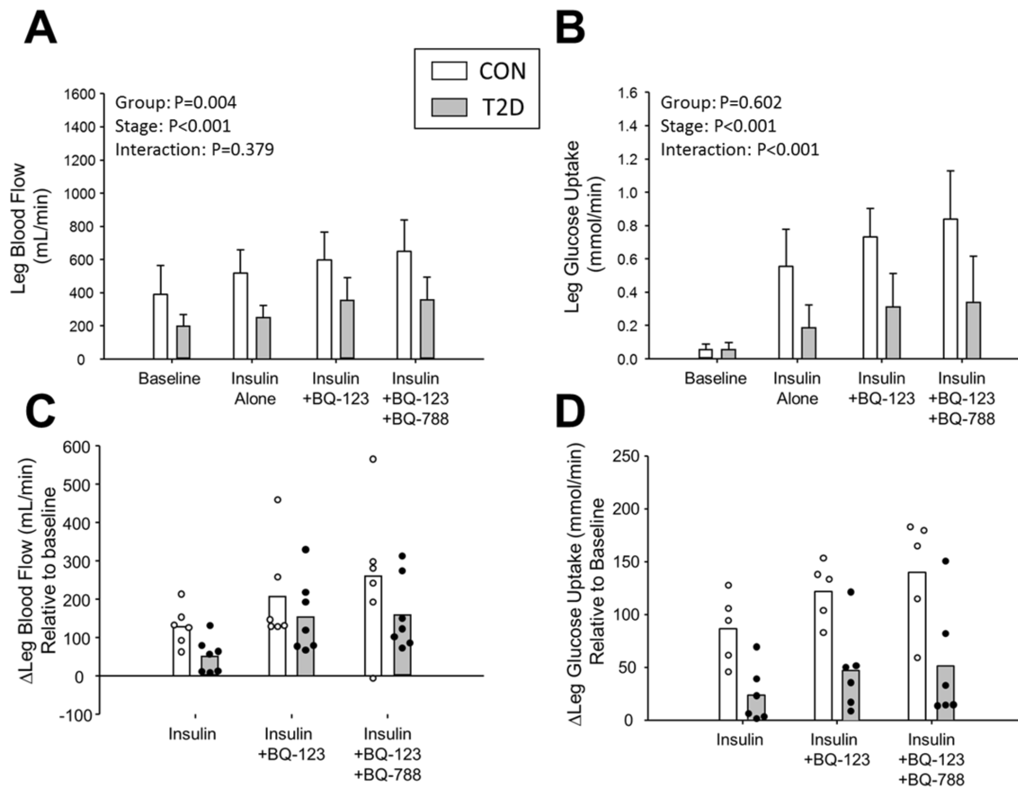

Figure 5. Panels A and B: Summary data for patients with Type 2 Diabetes (T2D; n=7) and control subjects (CON; n=7) showing absolute value leg blood flow (panel A) and leg glucose uptake (panel B) at baseline, during the insulin clamp (insulin alone), during the insulin clamp with intra-arterially infused Endothelin-1 A receptor antagonist BQ-123 (insulin + BQ-123), and during the insulin clamp with intra-arterially infused BQ-123 + the Endothelin-1 B receptor antagonist BQ-788 (insulin + BQ-123 + BQ-788). Panel C depicts the change in in leg blood flow in response insulin alone, insulin + BQ-123, and insulin + BQ-123 + BQ-788 relative to baseline between groups. Panel D similarly depicts the change in leg glucose uptake following insulin alone, insulin + BQ-123, and Insulin + BQ-123 + BQ-788 application relative to baseline values between groups.

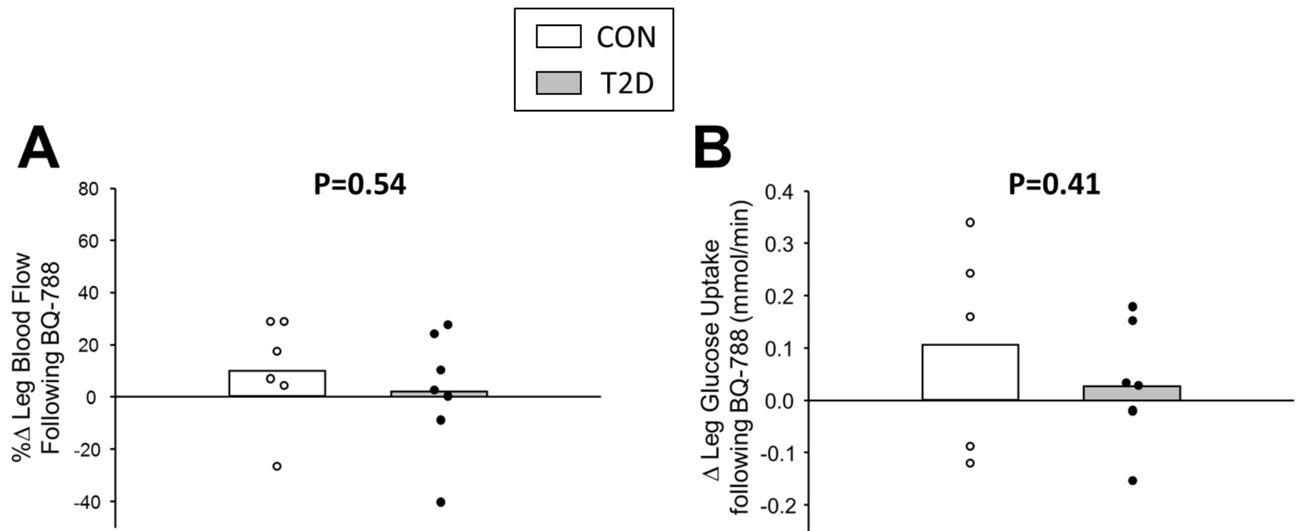

Figure 6. Panel A demonstrates a significant percent increase in response to BQ-788 (insulin + BQ-123 versus insulin + BQ-123 + BQ-788) in the patients with Type 2 Diabetes (T2D) compared to control (CON) subjects. Panel B demonstrates the change in leg glucose uptake, similarly, which was not different between groups. * $P<0.05$ patients with T2D vs. CON.

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Chapter 6

Conclusion

Throughout this dissertation we have provided evidence to support the notion that peripheral vascular dysfunction in patients with Type 2 Diabetes (T2D) is a causal factor contributing to the high incidences of hypertension (HTN) and (often subsequently) cardiovascular disease in these patients. Further, we provided rationale for the basis of why insulin itself, and the development of insulin resistance (IR) might plausibly underlie the impairment in vascular function in this population. Based on preclinical animal model studies, and human models of IR (e.g., obesity), this dissertation sought to elucidate the role that IR-induced alterations in both the central and peripheral actions of insulin may play in T2D-associated vascular dysfunction.

In Chapter 3 we demonstrated that patients with T2D exhibit augmented vasoconstrictor and blood pressure (BP) responses to spontaneous bursts of muscle sympathetic nerve activity (MSNA) under resting conditions (i.e., sympathetic transduction). Moreover, in Chapter 4 we provided evidence that hyperinsulinemia itself, does not cause augmented sympathetic transduction to BP. These findings may have important implications for BP regulation in patients with T2D, and as discussed in Chapter 3, enhanced sympathetic transduction to BP may represent a pathogenic link between T2D and hypertension (HTN). Beyond demonstrating augmented sympathetic transduction, in Chapter 5 we demonstrated that exaggerated endothelin-1 (ET-1) vasoconstrictor tone limits insulin-stimulated blood flow (BF) in patients with T2D, and that this effect is mediated predominantly through the ET-1 A (ETA) receptor rather than the ET-1 B (ETB) receptor. Yet, while we have provided evidence for novel mechanisms of peripheral vascular dysfunction, the studies within this dissertation have produced several lines of future investigation that warrant discussion.

First, it was interesting that cross-sectional comparison of patients with T2D currently taking statin therapy versus those who were not taking statins suggested statin therapy may be

capable of attenuating the enhanced sympathetic transduction to BP in T2D. Prospective follow-up studies (i.e., randomized placebo controlled cross-over clinical trials) are warranted to confirm that this effect is indeed related to statin therapy. Further, studies attempting to identify the mechanisms by which enhanced sympathetic transduction to BP manifests itself in these patients are also warranted.

In Chapter 4, we demonstrated that hyperinsulinemia itself, does not cause enhanced sympathetic transduction. However, studies translating these findings to patients with overt T2D are necessary. Important to this, a sympatho-attenuating action of local vascular insulin signaling is lost in patients with essential HTN (1), and this appears to be mediated by enhanced free radical production and the subsequent attenuation of nitric oxide (NO) bio-activity (2). Therefore, it is plausible given the proposed shift towards enhanced ET-1 production, and reduced NO production by vascular insulin signaling that is associated with insulin resistance [IR; (3)], patients with T2D might also display a loss of the sympatho-attenuating action(s) of insulin. If this were to be the case, hyperinsulinemia may then play a causal role in augmenting sympathetic transduction to BP in patients with overt T2D. To date, no studies into sympathetic transduction during hyperinsulinemia have been completed in this patient population. Further, the role that ET-1, independent of NO, plays in augmented sympathetic vasoconstriction has also been scarcely examined in humans, and thus might reasonably be the topic of future investigation.

Finally, in Chapter 5 we demonstrated an improvement in insulin-stimulated BF following ET-1 receptor antagonism in patients with T2D. Indeed, ETA receptor antagonism corrected ~50% of the impairment in insulin-stimulated BF in patients with T2D compared to control subjects, with minimal additive effect of ETB receptor antagonism. Therefore, studies might reasonably aim to determine the mechanisms underlying the remaining impairment in insulin-stimulated BF. Based

on Chapters 3 and 4, the sympathetic nervous system might reasonably be implicated in contributing to the persistent impairment in insulin-stimulated BF. However, no studies have even measured MSNA during experimental hyperinsulinemia in patients with T2D. Future studies in this area are requisite to begin to understand the role that the sympathetic nervous system plays in impaired insulin-stimulated BF in patients with T2D. Beyond those findings, despite an augmented improvement in insulin-stimulated BF following ETA antagonism during hyperinsulinemia in the patients with T2D, the increase in LGU was similar compared to controls. As discussed within Chapter 5, a plausible interpretation is that significant metabolic derangement persisted in the patients with T2D, such that despite augmented delivery of substrate to skeletal muscle, membrane permeability to glucose remained low. In that scenario the dependency on BF to maintain interstitial glucose concentration may be reduced, minimizing the beneficial effect of improving insulin-stimulated BF. Subsequent studies in patients with T2D, such as those completed recently in healthy humans (4) to calculate membrane permeability to glucose during insulin stimulation might reasonably confirm this hypothesis.

Collectively, the work contained herein supports role(s) for both greater sympathetic (via way of greater sympathetic transduction), and ET-1 mediated vasoconstriction in T2D-associated vascular dysfunction.

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