The Regulation of Release of Dipeptidyl Peptidase IV from Healthy and Diabetic Skeletal Muscle

by

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Abstract

Dipeptidyl peptidase IV (DPP-IV) is a multifaceted enzyme that can be shed from skeletal muscle membrane via matrixmetalloproteinases (MMPs). Tissue inhibitors of MMPs (TIMP) within the extracellular matrix (ECM) modulate the activation of MMPs. Hyperglycemia was shown to alter ECM proteins, likely causing a disruption in the shedding of DPP-IV from the membrane. However, little is known regarding the shedding of DPP-IV from skeletal muscle with stimulation and what role ECM proteins play in the mechanism. Additionally, it is not well known how hyperglycemia affects this system. In Part 1 of the study, C_2C_{12} skeletal muscle cells were stimulated following differentiation. To determine the role of ECM proteins, MMP2 was inhibited in one set and MMP9 was inhibited in another for 6 hours prior to stimulation. For Part 2, C_2C_{12} cells were exposed to normal (5mM), pre-diabetic (15mM), or diabetic (25mM) glucose conditions for 1, 7, or 10 days. At 10 days after differentiation, cells were stimulated and fractionated. DPP-IV released into the media was determined via fluorometric assay of pre and post media samples. MMP2, pro-MMP9, TIMP2, and DPP-IV of the membrane fraction of the cells were measured via ELISAs. In Part 1, no statistically significant changes with stimulation were seen except for the significant decrease in pro-MMP9 when cells were treated with an MMP9 inhibitor. In the second part, there were no significant differences in the DPP-IV shed following stimulation. MMP2 was increased in 25mM glucose after 7 and 10 days of exposure, pro-MMP9 was not significantly changed, TIMP2 was decreased after 1 and 7 days of exposure to 15mM glucose, and membrane bound DPP-IV was not significantly altered with glucose

exposure. From this study, it remains unclear if DPP-IV is shed from skeletal muscle; however, it was shown that ECM proteins play a role in the process and can be altered with hyperglycemia.

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List of Abbreviations

Ach	Acetylcholine
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
Akt	Protein Kinase B
AMPK	AMP-activated protein kinase
ATP	Adenosine Triphosphate
BMI	Body Mass Index
Ca^{2+}	Calcium
CDC	Center for Disease Control
cGMP	Cyclic Guanosine Monophosphate
CVD	Cardiovascular Disease
DM	Differentiation Media
DMSO	Dimethyl Sulfoxide
DPP-IV	Dipeptidyl Peptidase IV
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
GFP	Green Fluorescent Protein
GLP-1	Glucagon Like Peptide 1
GM	Growth Media
GTP	Guanosine Triphosphate

HUVEC	Human Umbilical Vein Endothelial Cells
IL-6	Interleukin 6
K+	Potassium
MMP2	Matrix Metalloproteinase 2
MMP9	Matrix Metalloproteinase 9
mRNA	Messenger RNA
MT-MMP	Membrane Type-MMP
NA	Noradrenaline
NGT	Normal Glucose Tolerance
NO	Nitric Oxide
NPY	Neuropeptide Y
O_2	Oxygen
RBC	Red Blood Cell
SDF-1	Stromal-Derived Factor 1
sGC	Soluble Guanylyl Cyclase
SIRT1	Sirtuin 1
SNA	Sympathetic Nerve Activity
TIMP2	Tissue Inhibitor of Matrix Metalloproteinases 2
TNF-α	Tumor Necrosis Factor α
WP	Whey Protein

Chapter 1: Introduction

Dipeptidyl peptidase IV (DPP-IV) is a serine protease that acts on several substrates throughout the body [1, 2]. There are two types of DPP-IV present in several locations including the blood, the kidneys, and the intestines. The soluble form is found mainly in the plasma and interstitial fluid, and the membrane-bound form can be found on T-cells, adipose tissue, vascular smooth muscle, and endothelium [2-4]. The function of DPP-IV that receives the greatest attention is its modulatory action on incretin hormones such as glucagon-like peptide 1 (GLP-1; **Figure 1**). GLP-1 is released from the gut after eating and targets the pancreas to stimulate insulin release [5]. DPP-IV acts to cleave GLP-1, rendering it inactive before it gets to the pancreas. Insulin secretion is ultimately diminished and resultant glucose uptake is negatively affected.

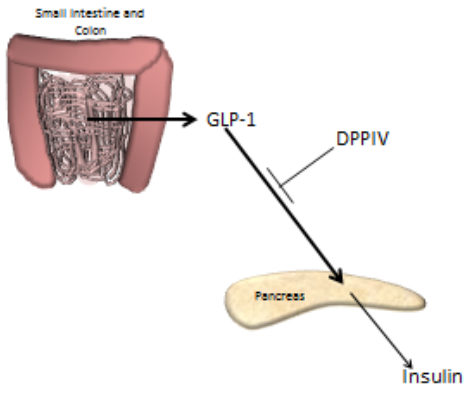

Figure 1. DPP-IV Interaction with GLP-1

Because of its interaction with GLP-1, DPP-IV has a growing link to Type 2 diabetes, as studies showed diabetics had higher DPP-IV activity levels than their matched controls [6]. As a result,

inhibitors of DPP-IV became a popular and proven pharmaceutical intervention for the management of diabetes [7, 8].

However, the exact physiology of how a regulatory enzyme becomes pathological was not well investigated. One idea was that fat tissue in obese people released more DPP-IV [9, 10]. This is a reasonable hypothesis given that obesity is a common comorbidity for Type 2 diabetes [11], albeit the link between high DPP-IV and obesity was based on BMI [12, 13]. The first study in a series of studies performed in the Kluess laboratory investigated the relationship of plasma DPP-IV activity with specific body composition measures in healthy individuals [14]. Based upon prior studies, we hypothesized that there would be a positive correlation between body fatness and DPP-IV activity. The results were unexpected in that our results demonstrated plasma DPP-IV activity was positively correlated with lean mass, but it was negatively correlated with fat mass in individuals. This finding led us to speculate as to whether DPP-IV originated from the muscle and/or whether higher DPP-IV in fit people with high muscle mass was physiologically beneficial.

Shortly after the conclusion of the aforementioned study, a study from another lab confirmed the release of DPP-IV from skeletal muscle [15] , which had implications for the findings from our body composition associations with DPP-IV. However, the purpose and mechanism for release was not as well investigated. As mentioned before, DPP-IV has several pleotropic roles, and some of these functions could be present at or near the skeletal muscle. Specifically, DPP-IV can be involved in cell proliferation via stromal derived factor 1 (SDF-1) and interleukin-6 (IL-6; [16-19] and insulin signaling via Akt phosphorylation [20]. One study

stated that the membrane-bound DPP-IV is the main source for the myokine evidenced by the findings that DPP-IV can be shed via matrix metalloproteinases (MMP; [21]. Therefore, the second study performed in the Kluess Laboratory investigated the production and release of DPP-IV from skeletal muscle in cell culture and rodent models [22]. The data from that study suggested that whey protein was able to robustly increase DPP-IV mRNA expression and secretion from muscle cells in vitro and the latter phenomena likely occurred via MMP2 and MMP9 present in whey protein and in the extracellular matrix (ECM). Interestingly, plasma DPP-IV was not changed in humans following an acute whey protein feeding or exercise bout, albeit muscle biopsy and/or microdialysis may have yielded similar results to our cell culture/rodent findings.

DPP-IV may also be involved with blood flow regulation to skeletal muscle through its proteolytic regulation of neuropeptide Y (NPY). In 1982, NPY was found to play a role in blood flow regulation as a powerful vasoconstrictor [23]. When left intact, NPY_{1-36} binds to the G-coupled protein Y1 receptor on the abluminal side of the vasculature and induces vasoconstriction either directly or via the potentiation of noradrenaline (NA) contraction [24, 25]. However, NPY can be cleaved to shorter sequences by DPP-IV and have an effect on different physiological processes (**Figure 2**). In regards to blood flow regulation, the shorter NPY_{3-36} plays an important role by having the opposite effect of its longer predecessor [26, 27]. NPY_{3-36} binds to Y2 receptors found prejunctionally or on the vascular smooth muscle and mediates vasodilation. A study by Evanson et al. demonstrated the effects of DPP-IV inhibition on the NPY overflow in the gastrocnemius first order arterioles of rats [24]. Specifically, these

authors reported that the presence of NPY was increased following stimulation of the arterioles, but this increase was augmented even further with the inhibition of DPP-IV. While a portion of the DPP-IV was found to be from the endothelium, there was still a large portion of the enzyme from other sources. The aforementioned findings suggest that DPP-IV from non-endothelial locales in the muscle arteriolar network could play a significant effect in blood flow regulation.

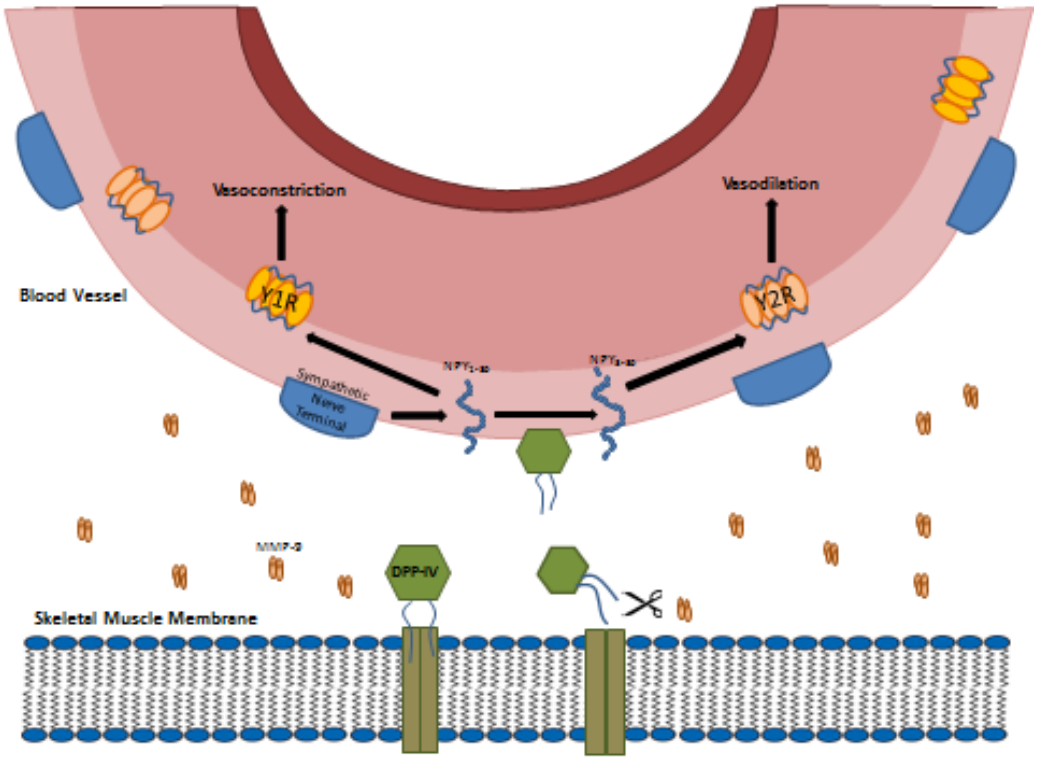

Figure 2. Role of DPP-IV in NPY-Mediated Vascular Response

In knowing whey protein was able to cause the shedding of DPP-IV from the sarcolemma, a third study from the Kluess Laboratory was developed to investigate the role of DPP-IV in blood flow regulation. Using intravital microscopy in a rodent model, we tested as to whether DPP-IV released from muscle can increase arteriolar diameter in the skeletal muscle

vascular bed via a reduction in NPY mediated vasoconstriction was investigated (unpublished results). Our findings suggested that DPP-IV was indeed released from the muscle and acted on NPY to attenuate vasoconstriction, which may be a mechanism for functional sympatholysis during exercise.

Hyperglycemia, especially leading up to the development of diabetes, impairs the vasculature's ability to regulate itself by attenuating nitric oxide (NO) production [28, 29]. Through several NO-based mechanisms, endothelial dysfunction becomes a major problem. Additionally at high levels of glucose exposure, the system of tissue inhibitors of MMPs (TIMP) is disrupted [30, 31]. Research showed MMPs are increased within the vasculature [31, 32] in diabetics, and together with altered TIMP, this could potentially lead to atherosclerosis and a disturbance in vascular remodeling, which contribute to the development of CVD [33].

The purpose of this study is two-fold: 1) to determine the role of MMP- and TIMP-mediated DPP-IV release during skeletal muscle contraction, and 2) establish how hyperglycemia and the development of diabetes alter this mechanism. The overall hypothesis is MMPs in the extracellular matrix will catalyze shedding of DPP-IV from skeletal muscle during contraction. Furthermore, I hypothesize that this mechanism will be negatively altered by exposure to high glucose via its effect on TIMP. The individual hypotheses are 1) when skeletal muscle is contracted/stimulated, membrane-bound DPP-IV will be shed from the membrane into the extracellular space via increased MMP activity; additionally, TIMP will alter the function of MMP, indirectly mediating the shedding of DPP-IV and 2) when skeletal muscle has been chronically exposed to high glucose concentrations, MMP2 and MMP9 will be increased within

the extracellular matrix, leading to excess shedding of DPP-IV, causing the system to be down regulated, and subsequently leading to the release of less DPP-IV during stimulation/contraction. Cell culture models will be used to investigate these hypotheses via imaging, cell fractionation, fluorescent assays, and ELISAs.

Chapter 2: Review of Literature

Blood Flow and Exercise Hyperemia

The cardiovascular system is comprised of many different parts that serve a multitude of functions throughout the body. Two key components of this system are the blood and the vasculature through which it flows. From delivering fuel for energy to removal of waste products, the blood exists as a transport system that without, the body cannot exist, and it is the responsibility of the vasculature to provide adequate transport at any given time to a specific region. For many years, the ability of the blood to increase and decrease flow to certain regions has been of interest to many people. However, it took many years to arrive at the conclusion that blood, was not only circulated, but that blood flow could be regulated.

The classical Greek philosopher, Galen of Pergamon, developed a theory about blood, and while it was incorrect, it was accepted as true for over 1000 years [34]. Specifically, he believed that two types of blood existed within the body: venous blood, which came from the liver, and arterial blood, that came from the heart. The venous blood carried the nutrients that the different organs needed, and arterial blood brought the vitality of life (air, chemicals, and heat) from the heart. Even more interestingly, it was proposed that blood simply ebbed and flowed throughout the body until it was either evaporated or consumed by other organs. Over the years, several scholars began to discover the incorrect teachings of Galen and found that blood was actually pumped through the heart and the vasculature originated from here, rather than the liver. By the 1600's William Harvey, basing his work off his predecessors' findings, was able to accurately depict that blood was conserved within the body and the heart acted as a pump for the

circulation of the blood [34]. Additionally, he was able to break down the difference in arterial and venous blood and flow, showing a dynamic regulation of the system. While all his findings were published in 1632 and further supported by Malpighi's detection of capillaries in 1661, it wasn't until the 1800's that the controversy surrounding Harvey's work would disappear, and his views accepted as physiological truth [34-36].

A Scottish surgeon named John Hunter made a statement in 1794 that laid the foundation for the discovery and research of blood flow regulation for over 200 years [37]. He simply stated "Blood goes where it is needed," which at the time was a profoundly curious and new observation, which would come to be known as hyperemia. Almost 100 years later, Walter Gaskell performed a study using the hindlimb of a dog to demonstrate the change in blood flow during and immediately following electrical stimulation of the muscle [38]. His study showed blood flow was increased immediately following the contractions and quickly returns to baseline without a second stimulation of the muscle. This study introduced exercise hyperemia, which was different from its precursors that investigated reactive hyperemia, increased blood flow caused by arterial occlusion, again raising the question of what causes vasodilation and presenting a new question of what role does the local muscle play in blood flow regulation [37-39].

Regulation of Skeletal Muscle Blood Flow

Skeletal muscle blood flow has several factors that are able to alter its state, whether in response to a simple activity as picking up a pen to write or a high functioning activity as

running a marathon. One factor that has been around since the discovery of hyperemia is the mechanical mechanism of the skeletal muscle pump [40, 41]. This mechanism can best be described as the constriction of vessels in the contracting skeletal muscle that upon release causes the vessels to open in a way that permits the suction of blood through the vessels, mimicking increased blood flow [42, 43]. While this phenomenon does occur within the skeletal muscle, it was found to not significantly contribute to exercise hyperemia. The skeletal muscle pump explains the question of how the immediate change in blood flow occurs at contraction, but it is limited by the sheer fact that prolonged and high force contractions impede blood flow [44, 45].

Chemical vasodilation is thought to be the most significant contributor to blood flow regulation. Metabolites that facilitate vasodilation are carried in the blood, found in the endothelium, or released by local muscle during contractions. With vast amounts of research taken place, numerous vasodilator substances have been identified and will be discussd in greater detail below. However, to date, there has not been a single chemical that explains the majority of the exercise hyperemia response. Resulting findings show many redundancies within the mechanisms, such that the response will be attenuated, but never abolished, when one substance is inhibited.

Potassium (K^+) channels in vascular smooth muscle have been found to play a potential role in the immediate response of exercise hyperemia [46]. These channels have four types, each having a different stimulus and a distinctive role in vasodilation. Voltage-dependent K^+ channels maintain and detect the membrane potential of the smooth muscle, and upon activation

(depolarization along the membrane), the channels open to prevent further depolarization from occurring [47]. Ca^{2+} activated K^+ (K_{Ca}) channels respond to alterations in intracellular Ca^{2+} concentrations [48]. As a moderator of myogenic tone, K_{Ca} channels open to allow an efflux of K^+ to counter any changes in the amount of vasoconstriction [49]. Inward rectifying K^+ channels detect the external hyperpolarization stimuli and prevent the loss of K^+ to the extracellular space [50, 51]. The final type of channel is the ATP-sensitive K^+ (K_{ATP}) channel. K_{ATP} channels are responsible for detecting changes in the metabolic state of the vascular smooth muscle and responding accordingly [52]. Most responses invoke vasodilation and can be inhibited by vasoconstricting substances. Several studies have shown that inhibition of any of these K^+ channels will cause a pronounced attenuation of vasodilation, leading to a decreased hyperemic response [52-54].

Over the years, hypoxia (decrease in oxygen) has been presented as a vasodilatory stimulus. Blood flow always matches the place that is taking up the most oxygen, so naturally, when there is a deficit, the vasculature is able to detect this state and increase the blood flow to that area to fight hypoxia. Many studies have demonstrated changes in blood flow in response to altered oxygen delivery, however most changes evoked are small in comparison to other vasodilating stimuli [55, 56]. While oxygen itself may not induce vasodilation directly, its detection is capable of altering the state of oxygen delivery via blood flow.

ATP was identified a vasodilator in the 1970's due to the appealing hypothesis that would link skeletal muscle contraction to exercise hyperemia [57]. Not only is ATP a marked metabolic substance, but also it is also readily available from numerous sources. Studies using exogenous

ATP have shown equivalent vasodilation responses as seen with corresponding exercise levels, in addition to decreases in Sympathetic Nerve Activity (SNA) [58-60]. ATP can be released as a cotransmitter with noradrenaline from sympathetic nerves into the neuromuscular junction, where it binds to P2X receptors on the external surface of vascular smooth muscle and signals for vasodilation [61-63]. Intraluminal ATP interacts with the endothelium by binding to P2Y receptors, resulting in vasodilation [59, 63].

While the effectiveness of ATP as a vasodilator is well established, the source of ATP during exercise is a highly debated topic. Red blood cells (RBC) are known to carry ~150mM ATP, making them a likely source for direct stimuli in response to mechanical deformation and hypoxia detection [64, 65]. The actual mechanism for ATP release from RBC, however, has created a controversy between two schools of thought. One hypothesis suggests that RBC detect hypoxic conditions in the exercising muscle and release ATP into the lumen of the vessel [64-66]. While this would make for a likely story, there are several limitations for the studies conducted, including the picomolar responses being measured. The other hypothesis is simply that any contributing ATP from RBC are simply products of mechanical deformation [67] and ultimately, hemolysis of the cells [68]. This seems to be a more valid approach, as the ATP concentration released from lysed cells is extremely high, making it more likely to induce a response.

Nitric oxide (NO) and prostaglandins are powerful vasodilators that are produced by the endothelium [69, 70]. Once released, NO enters the neighboring smooth muscles cells and activates soluble guanylyl cyclase (sGC) that is sensitive to NO detection. sGC catalyzes the

reaction taking guanosine triphosphayte (GTP) to cyclic guanosine monophosphate (cGMP), thereby activating Protein Kinase G. This cascade leads to increased Ca^{2+} uptake by the sarcoplasmic reticulum and signaling for relaxation of the smooth muscle, which is synonymous with vasodilation [69]. Studies using NO inhibition have shown decreases in hyperemia responses in human subjects, and when paired with inhibitors of other substances, little to no increase in the effect was seen [71-73]. These findings indicate that NO is somehow involved in the vasodilation mediated by many substances, making NO a key component in exercise-induced hyperemia. However, the decreases seen with NO inhibition are ~20% [39], maintaining the earlier premise that no one substance has been identified as the sole contributor to exercise hyperemia.

Finally, neural mechanisms have some of the oldest roots in their research as F. Pourfois du Petit discovered vasomotor nerves in 1727, shortly after Harvey's work was published [37]. As part of the autonomic nervous system, sympathetic nerves innervate skeletal muscle in order to modulate vascular tone. By releasing noradrenaline from the end terminals, sympathetic nerve activity (SNA) is able to bind to post-junctional receptors and maintain a constant tone of the vasculature through vasoconstriction [74]. When in need, SNA will cause an increase or decrease in vasoconstriction as to narrow or widen the vessel, changing blood flow and blood pressure. Additionally, much research has been done, and while it was originally proposed that sympathetic vasodilator nerves existed, that does not seem to be the case [75-77].

A secondary neural mechanism was proposed, but akin to the sympathetic vasodilator nerve theory, it too was found to not be a likely contributor to blood flow modulation. Spillover

of acetylcholine (Ach) from skeletal muscle motor nerves would match changes in vascular tone in conjunction with muscle contraction [78]. This was quickly reduced to a minor contributor, if one at all, by several studies showing no vasodilation with Ach release in the absence of muscular contraction [39, 79, 80].

Sympatholysis and NPY

With both possibilities of specific nerve activity causing vasodilation being false, a newer, more attractive theory arose known as functional sympatholysis [81]. SNA is constantly in action, working to keep vascular tone at a level that preserves resting blood pressure. This new hypothesis suggests that with exercise hyperemia, SNA is decreased to such a level that vascular tone is relaxed, creating the appearance of vasodilation [82]. The term, originally coined in the 1960's, was established in a study investigating the blood flow to the hindlimb of a dog [81]. In the control condition, a decrease in perfusion pressure was observed when contractions were stimulated in the skeletal muscle. However, when carotid occlusion, an SNA stimulus, was imposed, perfusion pressure increased at rest, but during contractions, the same response to the level of perfusion pressure occurred. This demonstrated that even with a sympathetic-evoking stimulus, SNA is overcome during exercise or when increased O_2 demand is required.
There have been many studies following the 1962 coining of the term that have further solidified this theory of functional sympatholysis [83-85]. One such study looked at the dose-response of exercise on sympatholysis [86]. Brachial artery blood flow was assessed during rest and two different exercise intensities under three different doses of tyramine, a drug known to release

noradrenaline from sympathetic nerves. At rest, there was a dose-response to the tyramine, showing increased constriction with increasing doses. However, an intensity dependent effect of exercise was shown to attenuate the vasoconstrictive ability of the tyramine.

Several studies have looked to determine how the mechanism for blocking noradrenaline binding to α- receptors occurs [87, 88]. NO made its way to be a front-runner in the discussion [89, 90], but studies quickly determined that it was not the sole player in functional sympatholysis. Exogenously applied NO was not able to inhibit sympathetic vasoconstriction [91], and in another study, NO-inhibitors were not able to diminish the exercise hyperemia response [92]. Even though, NO is not likely to be a major contributor, there is a possibility that it acts with other substances to facilitate functional sympatholysis in working skeletal muscle. Other likely candidates for mediators were hypoxia and ATP, as evident in their ability to promote exercise hyperemia as discussed previously.

Neuropeptide Y (NPY) is a 36- amino acid polypeptide that acts as a neurotransmitter of the sympathetic nervous system [74]. Co-released with noradrenaline and ATP from sympathetic end terminals, it produces vasoconstriction upon the local vasculature via activation of the Y1 receptors. This is a consistent interaction between these players at rest in order to maintain vascular tone. However, in the presence of exercise, NPY release was shown to increase, but have different effects in different areas of the body. It is evident that peripheral SNA is increased in order to redistribute the blood flow from unnecessary areas such as the gastrointestinal system to provide the active muscles with more blood. Locally, some SNA occurs during exercise to

prevent a drop in blood pressure by preventing excess hyperemia and keeping a steady vascular resistance [93].

Buckwalter et al. reported studies that explored NPY-vasoconstriction during exercise in the active skeletal muscle [94, 95]. Accordingly, these authors demonstrated that activation of NPY Y1 receptors during exercise produces vasoconstriction; however this vasoconstriction was reduced as the intensity of exercise increased. Several other factors were also explored during these experiments. One of note was the finding that NO did not appear to be accountable for this reduction in vasoconstriction as previously suggested. These results support the idea that during exercise, SNA is used to regulate vascular tone, but as intensity increases, sympathetic NPY is playing a lesser role.

NPY, DPP-IV, and Skeletal Muscle Blood Flow

Studies looking at NPY release at the vascular level rather than whole body showed a direct relationship between the amount of stimulation received and the amount of NPY that was released. Furthermore, there is evidence that a high intensity stimulus is required to elicit an increase in NPY from the sympathetic end terminals, and not just noradrenaline. Evanson et al. demonstrated a significant increase in NPY overflow from isolated arterioles immediately and 30 seconds following high frequency stimulation [24]. However, this increase was dependent upon dipeptidyl peptidase IV (DPP-IV). DPP-IV is a protease that cleaves the proline residue located near the N-terminus of NPY [2]. This takes NPY from its full form, NPY_{1-36}, to a truncated form, NPY_{3-36}, also altering the selected receptor type for NPY. Rather than binding to the Y1 receptor

on the vascular smooth muscle to cause vasoconstriction, NPY_{3-36} binds to pre-junctional Y2 receptors and limits further NPY release from the terminal [26, 27]. As a result, DPP-IV is able to alter NPY-mediated vasoconstriction by decreasing the amount of Y1-agoanist NPY_{1-36} available, in addition to signaling for less NPY release via activation of Y2 receptors.

Interestingly, the source of DPP-IV has been investigated and found to be present in numerous locations throughout the body, including as a soluble form that freely flows in the blood. For several years, it has been well established that DPP-IV is present on the endothelium of blood vessels [2, 6]. However, only recently has it been discovered that DPP-IV is present on the surface membrane of skeletal muscle and can be released as an additional source of interstitial DPP-IV [15]. Upon inhibition of DPP-IV, an increase in NPY was seen from stimulated arterioles indicating that DPP-IV can potentially play a role in skeletal muscle blood flow through its modulation of NPY [24]. A study using a technique known as intravital microscopy was able to investigate this mechanism in the gluteus muscle of a rat (unpublished results). When DPP-IV was released from the muscle, blood flow was increased in the experimental muscle. However, when DPP-IV was inhibited, the increase in blood flow was abolished. When looking at this data, it suggests that this relationship between DPP-IV and NPY could be a mechanism behind functional sympatholysis.

Several studies have showed increased DPP-IV mRNA expression following stimulation of skeletal muscle cells and exercised gastrocnemius muscles of rats [15, 22]. However, there have been no studies to date that have shown DPP-IV released from skeletal muscle in response to exercise. One study that investigated this question found no significant increase in DPP-IV

released from the muscle [15], but DPP-IV was measured after at least 4 hours of chronic stimulation of the muscle cells and not an acute bout. However, a recent study in the Kluess Lab showed that local plasma DPP-IV is increased following an intense bicep curl protocol in the arm, but remained unchanged in the control arm (unpublished results). It is unclear, though, from where this DPP-IV was released, albeit it is likely that it originated from skeletal muscle given that venous outflow from the biceps brachii coalesces at the median cubital vein (site of blood draw).

Matrix Metalloproteinases

DPP-IV exists on skeletal muscle as a Type 2 membrane-bound protein and is released by a process known as shedding [96], which was demonstrated to occur via matrix metalloproteinases (MMPs) [21, 22]. MMPs localized in the extracellular matrix (ECM) are calcium- and zinc-dependent endopeptidases that act to digest many ECM proteins and are predominantly associated with inflammation and tumor development, specifically aiding in angiogenesis [97, 98]. In 2014, Rohrborn et al. demonstrated for the first time the shedding of DPP-IV from the membrane of adipocytes and smooth muscle cells via MMPs [21]. Specifically, MMP1, MMP2, and MMP14 contributed to shedding from SMCs, where MMP9 was found to generate adipocyte shedding of DPP-IV. Neidert et al. showed the same mechanism occurring on skeletal muscle cells, via MMP2 and MMP9 [22]. However, with its interaction with DPP-IV, it is possible MMPs may play a role in exercise-induced hyperemia.

Studies have shown immediate increases in MMP9 following exercise bouts. A European group studied several pre-inflammatory markers in 22 half-marathon and 18 marathon participants [99]. Within 15 minutes after completing their race, plasma IL-6, TNF-α, and MMP9 were all significantly increased, with the greater increases seen in the full marathon runners, but all values returned to pre-race levels within 24 hours. Another group showed the increase in plasma MMP9 occurring 5 minutes after the end of a cycling bout [100]. While this increase in MMP9 is mostly attributed to the inflammatory response, it is possible that it additionally acts in conjunction with other pathways including the shedding of DPP-IV by the muscle. Furthermore, these measures were made in the circulating blood, which does not elicit a localized measure from the muscle.

While MMPs seem to be readily available in the ECM, they must first be activatedin order to possess catalytic activity [101]. Most pro-MMPs (inactive MMPS) are activated by a stepwise process which involves several proteolytic cleavage steps that convert the inactive pro-MMPs to their activated MMP form [102]. Activation of this mechanism can be caused by several factors including NO, heat, and low pH, all of which occur locally in the muscle during exercise. Pro-MMP-2 activation can only occur via cell surface activation [103]. In this process, membrane-type MMPs (MT-MMPs) are responsible for the cleavage that activates the proteinases. Some require the assistance of tissue inhibitors of metalloproteinases (TIMPs) [104], where some are able to directly generate activation [102].

Tissue Inhibitors of MMP

In the regulation of MMP biological activity, TIMPs are the select inhibitors that are able to directly control the level of MMP activity within the ECM [102, 105]. There are four different TIMPs, each with their own specific inhibitory properties. Additionally, each of the isoforms is activated and responds to different physiological and pathological conditions [106]. As a result, any alteration of TIMP levels will directly affect those of MMPs, and this can be seen in exercise. After a maximal treadmill exercise protocol, healthy individuals experienced an increase in circulating TIMP1 and TIMP2, but no significant change in MMP2 or MMP9 [107]. However, the aforementioned study reported very modest positive correlations between TIMP1 and MMP9 and between TIMP2 and MMP2. The correlation between TIMP2 and MMP2 is plausible as TIMP2 is required for the stepwise activation of MMP2 [104], but TIMP1 being positively correlated with MMP9 is not well understood. This could be due to multiple factors, including the sample was in circulating blood and not a local measurement from the muscle. Therefore, the measurement of TIMP and MMP within and near the exercising muscle ECM needs to be investigated.

Cardiovascular Disease, Diabetes, and Blood Flow

In the overall regulation of blood flow, neural, chemical, and mechanical factors have all been identified, and it seems that there are numerous redundancies between all of these factors to ensure the proper regulation occurs, even when one mechanism fails [39]. However, during the development of diseases, it can be more than one system that is affected causing drastic

responses. Cardiovascular diseases exhibit impairments in the regulation of blood flow through several mechanisms in addition to blockages of the vessels [108]. As a result, an individual may suffer from heart attacks, strokes, heart failure, and other problems with the cardiovascular system. Currently, the Center for Disease Control (CDC) reports cardiovascular disease (CVD) as the leading cause of death in the United States, with approximately 1 in every 4 deaths being related to heart disease.

In a normal state, the vasculature is dynamic in that it constantly remodels itself in response to different stimuli, allowing it to adapt. However, in CVD, the stimuli to which it is repeatedly exposed causes the structural integrity of the vessels to be compromised [109]. For example, vessel walls become stiffened, meaning they do not easily relax and constrict to maintain vascular tone, or the vessels form atherosclerotic plaques that reduce the area available for blood to freely flow [110]. Some of this has been attributed to MMPs and their role in vascular remodeling. In the development of plaque, several cytokines are produced which are known to increase the synthesis of MMPs in the ECM of smooth muscle cells and endothelial cells [106]. Over time, MMPs signal for the migration and proliferation of cells into the ECM, and in a diseased state, results in atherosclerotic plaque [111-113]. However, there remains little evidence that MMPs are the prime mediator of the plaque formation.

There are several risk factors that contribute to cardiovascular events including obesity, smoking, and physical inactivity [114]. Diabetes is another risk factor given that chronic exposure to high levels of glucose causes a disruption in multiple facets of the hyperemic response [29, 115]. The CDC reported in 1997 that more than 30% of diabetics in the US aged

35 years or older have a history of some cardiovascular event, and this statistic has not changed in almost 20 years despite research and heightened awareness. Type 2 diabetes accounts for approximately 90% of the 29.1 million diabetics in the US and is commonly brought on by poor diet and low levels of physical activity.

In a poorly regulated and high glucose diet, the body becomes resistant to insulin which in turn, causes the pancreas to increase the amount of insulin it is secreting. However, this system is eventually exhausted to the point where the pancreas can no longer make enough insulin to meet the demand. This results in chronic overexposure to high levels of circulating blood glucose, which becomes a cause of vascular dysfunction in the body among other things. Some of this dysfunction is endothelial dependent as hyperglycemia was shown to decrease NO originating from the endothelium, resulting in decreased vasodilation [116]. Other mechanisms affect the smooth muscle cells of the vessels [117]. In diabetics, the function of the sympathetic nervous system is diminished [118], and the production of reactive oxygen species is increased [119].

Similar to CVD, the production of MMPs is increased, leading to migration and proliferation of smooth muscle cells and disruption of the vasculature. In 2003, Death and colleagues exposed both Human Umbilical Vein Endothelial Cells (HUVECs) and macrophages to normal and high concentrations of glucose [31]. They reported that MMP1 and MMP2 were increased in HUVECs after the exposure to high glucose, while MMP3 was reduced. MMP9 did not change in endothelial cells, but showed an increased activity and expression in macrophages. A few years later, Ho et al. demonstrated similar findings in HUVECs treated with normal and

high concentrations of glucose [32]. Following 48 hours of exposure, activated pro-MMP2 was increased from control conditions and TIMP2 was decreased at several time points. MMP9 and TIMP1, however, were not shown to change in that time period.

In addition to the findings above, several other studies have established that high glucose exposure disrupts the balance of MMP and TIMP in vascular cells [120, 121]. When looking at muscle, vascular smooth muscle cells are commonly researched. However, little evidence exists on the disturbance of this system in skeletal muscle. A recent study by Kang et al. reported that in control fed mice, deletion of the MMP9 gene did not cause insulin resistance [122]; however, in high-fat diet mice, the deletion of the MMP9 gene exacerbated the degree of insulin resistance in the skeletal muscle. These findings convey the importance of MMPs in the regulation of the ECM of skeletal muscle; however, increased glucose exposure can potentially increase the enzymes above normal levels and lead to several complications as demonstrated by these findings in other tissues.

One of these complications could potentially be increased release of DPP-IV from the skeletal muscle membrane. This would support several findings that individuals with Type 2 diabetes have significantly higher plasma DPP-IV than their BMI-matched normal glucose tolerant (NGT) controls. Interestingly, one study showed that plasma DPP-IV of Type 1 diabetics was not different from their BMI-matched NGT controls [6] indicating that there is a difference in the pathophysiology of diabetics and how this affects circulating DPP-IV. What is more interesting are the findings of this study and a another study from the Kluess lab showing the plasma DPP-IV activity levels of NGT individuals with apparently healthy BMIs is higher than

the DPP-IV activity of those individuals that were overweight and/or Type 2 diabetics [6, 14]. Further investigation showed that plasma DPP-IV activity is positively correlated with lean body mass and negatively correlated with fat mass. These findings suggest that in healthy individuals with a normal BMI, higher DPP-IV activity is healthy for normal physiological function, and in the presence of Type 2 diabetes and overweight individuals, this system is disrupted and down-regulated. Following the increased MMP activity with glucose exposure, this over-stimulation of DPP-IV release could potentially cause this down-regulation of the system to decrease the amount of membrane bound DPP-IV. As a result, there are fewer enzymes available to be released and interact with NPY to alter its length and allow for decreased vasoconstriction during exercise.

The impairment of functional sympatholysis is evident in the presence of disease. Hypertensive patients were reported to have lower blood flow to their exercising muscles in addition to increased systemic blood pressure when compared to healthy controls [109]. In the same study, it was further demonstrated that this diminished blood flow was likely due to impairments of sympatholysis and not a reduced capability to form NO within the vasculature. Functional sympatholysis also appears to be compromised in diabetes, however to what degree and how remains unclear. One study reports that endothelial dysfunction must coexist to cause the changes [123], where others have shown endothelial-independent findings of reduced response of smooth muscle cells to NO stimulus [116, 124]. What is clear, however, is that there exist structural and functional changes that occur and could have an effect on the hyperemic

response during exercise, and the disruption of the DPP-IV-MMP-TIMP system following increased glucose exposure could potentially be one of these changes.

Chapter 3: Journal Manuscript

Introduction

Dipeptidyl peptidase IV (DPP-IV) is a serine protease that serves many roles throughout the body including in the gut, immune system, and vasculature [1, 2]. Existing in two forms, it can be found as a soluble enzyme in plasma and interstitial fluid whereas the membrane bound form is located on T-cells, muscles and endothelium [2-4]. One of the primary roles of DPP-IV within the vasculature is its modulation of neuropeptide Y (NPY) [2]. The intact version, NPY_{1-36}, acts on abluminal Y1 receptors to signal vasoconstriction of the vasculature as a mediator of blood pressure [24, 25]. However, DPP-IV shortens NPY to NPY_{3-36}, which no longer binds to Y1 receptors, but rather Y2 receptors, allowing for relaxation of the vascular smooth muscle and thereby increasing blood flow to that area [26, 27]. Evanson et al. demonstrated that following stimulation of arterioles, NPY was increased [24]. But upon the inhibition of DPP-IV, this increase was augmented, suggesting that DPP-IV plays a role in the regulation of NPY and vascular tone.

While some DPP-IV is released from endothelial cells, it is also present on the outside of the vasculature from a nearby source. The shedding of DPP-IV from the skeletal muscle membrane has been demonstrated in both cell culture and rodent models and is likely a source of DPP-IV at or near the vasculature [15, 22]. Upon activation, matrix metalloproteinases (MMPs) in the extracellular matrix (ECM) cause this release of DPP-IV from the membrane. Specifically, MMP2 and MMP9 were shown to be the key players in this process [21, 22]. Additionally, tissue

inhibitors of MMPs (TIMP) regulate the activation of MMPs, and by in part, play a key role in the shedding of DPP-IV.

Hyperglycemia, especially leading up to the development of diabetes, impairs the vasculature's ability to regulate itself by attenuating nitric oxide (NO) production [28, 29]. Through several NO-based mechanisms, endothelial dysfunction becomes a major problem. Additionally at high levels of glucose exposure, the system of TIMP is disrupted [30, 31]. Research showed MMPs are increased within the vasculature in diabetics [31, 32], and together with altered TIMP, this could potentially lead to atherosclerosis and a disturbance in vascular remodeling, which contribute to the development of CVD [33].

The purpose of this study is two-fold: 1) to determine the role of MMP- and TIMP-mediated DPP-IV release during skeletal muscle contraction, and 2) establish how hyperglycemia and the development of diabetes alter this mechanism. The overall hypothesis is MMPs in the extracellular matrix will catalyze shedding of DPP-IV from skeletal muscle during contraction. Furthermore, I hypothesize that this mechanism will be negatively altered by exposure to high glucose via its effect on TIMP. The individual hypotheses are 1) when skeletal muscle is contracted/stimulated, membrane-bound DPP-IV will be shed from the membrane into the extracellular space via increased MMP activity; additionally, TIMP will alter the function of MMP, indirectly mediating the shedding of DPP-IV and 2) when skeletal muscle has been chronically exposed to high glucose concentrations, MMP2 and MMP9 will be increased within the extracellular matrix, leading to excess shedding of DPP-IV, causing the system to be down regulated, and subsequently leading to the release of less DPP-IV during stimulation/contraction.

Cell culture models will be used to investigate these hypotheses via imaging, cell fractionation, fluorescent assays, and ELISAs.

Methods

Cell Culture

C_2C_{12} myoblasts were grown on 60mm plates in Growth Medium [GM; Dulbecco's Modified Eagle Medium (DMEM), 10% (vol/vol) fetal bovine serum, 1% penicillin/streptomycin, 0.1% gentamycin] at a seeding density of 3.5 x 10^5 at 37°C in a 5% CO_2 atmosphere. Forty-eight hours after myoblast growth reached 80-90% confluency, differentiation was induced by replacing GM with Differentiation Medium [DM; DMEM, 2% (vol/vol) horse serum, 1% penicillin/streptomycin, 0.1% gentamycin]. For 10 days following differentiation induction, DM was replaced every 24h to allow for myotube growth.

Part 1: Modulation and Release of Skeletal Muscle DPP-IV via MMP-TIMP Mechanisms in Cell Culture following Electrical Stimulation

Cell Culture Treatment and Inhibition of MMP

For Part 1, 10 plates, each containing 6-wells, were utilized. The five conditions included Control-No Stimulation (CTL), Control-Stimulation (CTL-Stim), MMP2 inhibitor (MMP2i), MMP9 inhibitor (MMP9i), and Whey Protein (WP). The whey protein plate was treated with 13μg/mL (100mmol/L) whey protein hydrolysate in the media 6 hours prior to stimulation. MMP-2 and MMP-9 inhibited experimental plates were treated with the corresponding inhibitors

in the media. MMP-2 inhibitor (12nm/L; EMD Biosciences, San Diego, CA) and MMP-9 inhibitor (5nmol/L; EMD Biosciences, San Diego, CA) were delivered to their respective plates via DMSO 6 hours prior to stimulation.

Electrical Stimulation and Fluorescent Imaging

On the day of the experiment, myotubes were removed from the incubator and maintained in DM during the experimental protocol. Cells were then moved to the stage of an Olympus IX71 inverted microscope equipped with an XM10 monochrome fluorescence CCD camera. To examine release of DPP-IV, the Green Fluorescent Protein (GFP) fluorochrome setting was used with excitation at 475nm and emission collected through a 509nm filter. Live-imaging was recorded for 15 seconds for baseline values then continued through the experimental trial until the fluorescence reached a steady state. For control cells, the imaging was recorded for 60 seconds.

Once a baseline measure was recorded, the experimental cells were electrically stimulated at a frequency of 1Hz for a duration of 2ms pulses at 5V. The stimulation continued for a total time of 5 seconds, during which the release of DPP-IV from the membrane was observed. The stimulation was repeated three more times with a 10 second break in between each bout. Cell culture media was collected before and after stimulation for DPP-IV activity assay and stored at -80°C until analysis.

Ultracentrifugation for Cellular Fractionation

The control and experimental cells were scraped and centrifuged at 300G for 10 minutes to isolate the cell pellet. The media was syphoned off, and the cells were fractionated using the Mem-PER Plus Protein Fractionation Kit (Thermo Scientific). Isolation of the cytosol and membrane proteins was completed following the manufacturer's instructions. Fractions were stored at -80°C until further analysis.

ECM Protein Determination

DPP-IV, MMP9, MMP2, and TIMP2 protein concentrations of the cell membrane fractions was determined using mouse DPP-IV, pro-MMP9, MMP2, and TIMP2 ELISA kits (Sigma-Aldrich, Inc., St. Louis, MO) per the manufacturer's instructions.

DPP-IV Activity Assay

The fluorometric DPP-IV assay developed by Scharpe was used to measure the activity of the cell culture media of the pre and post conditions [125]. The assay measures the release of 4-methoxy-2-naphthylamine to determine the activity of DPPIV enzymatic activity. Enzymatic activity was defined as the amount of DPP-IV that cleaves 1µmol of glycyl-L-proline-4-methoxy-2-nephthylamine per minute. DPP-IV activity was calculated using the equation below:

Activity (U/L)= [(S) x VA x 1000 x Cst)]/ [(T x Sv) x (F)]

Where S is the sample fluorescence minus the sample blank fluorescence, VA is the total assay volume, 1000 is the correction factor for milliliters to liters, Cst is the standard concentration, T

is the incubation time, Sv is the sample volume, and F is the standard florescence minus the standard blank florescence.

Total protein concentration of the media samples was determined by Coomassie Protein Assay following the manufacturer's instructions.

Part 2: Modulation and Release of Skeletal Muscle DPP-IV via MMP-TIMP Mechanisms following Electrical Stimulation in Cell Culture Chronically Exposed to Glucose

Cell Culture Treatment

For Part 2, 14 plates, each containing 6-wells, were utilized. Upon introduction of differentiation, cells were exposed to three different glucose concentrations for different time points across 10 days. For normal glucose exposure, the cells were treated with 5mmol/L glucose. This was considered the control and was used in all cells until treatment with higher concentrations occurred. Pre-diabetic cells were treated with 15mmol/L glucose, and cells exposed to diabetic conditions were treated with 25mmol/L glucose. To investigate the development of diabetes and how it affects the systems of interest, cells were treated for 1, 7, or 10 days in their respective glucose concentrations.

Electrical Stimulation and Fluorescent Imaging

On the day of the experiment, cells were removed from the incubator and kept in DM throughout the stimulation process. Cells were moved to the stage of an Olympus IX71 inverted microscope equipped with an XM10 monochrome fluorescence CCD camera. To examine

release of DPP-IV, the same protocol as above was used. Cell culture media was collected before and after stimulation for DPP-IV activity assay and stored at -80°C until analysis.

Ultracentrifugation for Cellular Fractionation

The cells were scraped and isolated as described above and underwent the same process of ultracentrifugation using the Mem-PER Plus Protein Fractionation Kit.

ECM Protein Determination

Cell membrane DPP-IV, MMP9, MMP2, and TIMP2 protein concentrations were determined as described above.

DPP-IV Activity Assay

DPP-IV activity and total protein concentration of the cell culture media samples were determined using as described in Part 1.

Statistics

All data is reported in **Tables 1 and 3** as mean ± standard deviation and the n for each group. For Part 1, a one-way ANOVA was used to determine which conditions were significantly different from the Control group. Tukey's post-hoc test was implemented to conclude specific conditions that were significant from each other if the overall ANOVA was found to be significant. For Part 2, a one-way ANOVA determined which groups were

significantly different from the CTL cells. If significance was found, Tukey's post-hoc was used to determine which conditions were significantly different from each other. All data was tested for violations of statistical assumptions. If any violations were found, a non-parametric Kruskal-Wallis test was used to determine statistical significance followed by t-tests with equal variances not assumed to determine specific differences between groups. Alpha level for the entire series was set at $p<0.05$. All statistics were run with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) and IBM SPSS Statistics (IBM Corporation, Armonk, NY). All F-values, degrees of freedom, and p values are reported in **Table 2 and 4**.

Results

Part 1

DPP-IV Release in Response to Stimulation

DPP-IV activity of the media was measured before and immediately after stimulation of the muscle cells. Due to the possible denaturing of proteins caused by stimulation, total protein concentration of each sample was determined to yield DPP-IV activity per protein. Compared to CTL cells, DPP-IV release was not significantly different following stimulation in the CTL-Stim cells (0.0702±0.1969U/µg vs. 0.0777±0.1438U/µg). DPP-IV release from MMP2i and MMP9i cells was decreased (0.0240±0.1211U/µg and 0.0185±0.1096U/µg), but this change was not significantly different from CTL or CTL-Stim cells (**Figure 3A)**. No change was found in the membrane DPP-IV concentrations (CTL: 835.6±302.9pg/ml; CTL-Stim: 831.9±220.7pg/ml; MMP2i: 854.5±263.2pg/ml; MMP9i: 890.1±335.9pg/ml; **Figure 3B**).

Alterations to Membrane Proteins

The membrane protein concentration of MMP2 was not significantly changed following stimulation of the CTL-Stim (84.74±32.73pg/ml) from CTL (101.5±69.37pg/ml; **Figure 4A**). When either MMP2 or MMP9 was inhibited, there was also no change in membrane MMP2 (MMP2: 103.1±51.22pg/ml and MMP9: 64.00±14.71pg/ml) from the CTL cells. Interestingly when MMP9 was inhibited, membrane MMP9 was significantly decreased (14.81±5.04pg/ml) following stimulation when compared to CTL cells (26.64±12.25pg/ml; p=0.0242; **Figure 4B**). There was no change in MMP9 following stimulation in CTL-Stim cells (17.77±11.91pg/ml), and no change occurred in MMP9 in MMP2i cells (28.61±11.88pg/ml). Membrane TIMP2 was not altered in the CTL-Stim cells (172.4±90.6pg/ml) compared to CTL cells (152.2±83.2pg/ml), and both the MMP2i (135.5±32.1pg/ml) and MMP9i cells (103.3±37.2pg/ml) saw no significant changes from CTL as well (**Figure 4C)**.

Part 2

DPP-IV Release in Response to Stimulation

After 1 day of increased glucose exposure to either 15mM or 25mM, DPP-IV released with stimulation was not changed (15mM: 0.0433±0.2015U/µg; 25mM: 0.0234±0.1175U/µg) compared to that released by the normal 5mM CTL cells (0.0777±0.1438U/µg). Similarly, DPP-IV release was still unchanged after 7 days of increased glucose exposure (15mM: 0.0046±0.0767U/µg; 25mM: 0.0511±0.1058U/µg). And following 10 days of increased glucose

exposure, the DPP-IV released remained similar (15mM: 0.0787±0.0706U/µg; 25mM: 0.0854±0.2282U/µg) to the values of the CTL cells. All data for DPP-IV shed following glucose exposure can be seen in **Figure 5A**.

Finally, membrane bound DPP-IV was found to not have any significant changes following glucose exposure (p=0.0840). DPP-IV at the membrane was not significantly changed after 1 day at 15mM and 25mM glucose (15mM: 728.20±224.40pg/ml and 25mM: 562.40±78.64pg/ml) compared to 5mM CTL (831.90±220.70pg/ml). At 7 Days, membrane DPP-IV remained similar to CTL in the 15mM and 25mM cells (15mM: 684.50±144.70pg/ml and 25mM: 667.60±236.20pg/ml). Both the 15mM and 25mM cells remained constant out to 10 days (15mM: 614.60±59.18pg/ml and 25mM: 583.80±146.30pg/ml) to levels that were not significantly different from CTL cells. All data for Membrane bound DPP-IV following glucose exposure can be found in **Figure 5B**.

Alterations to Membrane Proteins following Increased Glucose Exposure

Analysis of MMP2 showed a significant change across the different conditions (p=0.0120; **Figure 6A**). Membrane MMP2 was not changed following 1 day of exposure to 15mM glucose (90.18±49.96pg/ml) or 25mM glucose (84.91±41.81pg/ml) from 5mM CTL cells (84.74±32.73pg/ml). After 7 days of exposure to increased glucose, MMP2 was still unchanged from CTL levels in the 15mM cells (63.18±27.37pg/ml); however in the 25mM cells, membrane MMP2 significantly increased (170.40±88.25pg/ml; p=0.0300). By Day 10 of increased glucose

exposure, 15mM cells (133.20±88.57pg/ml) were still similar to CTL cells while 25mM cells (233.70±161.90pg/ml) saw membrane MMP2 levels above the 5mM CTL cells (p=0.0500).

Membrane MMP9 saw a significant difference among the groups (p=0.0280; **Figure 6B**). Cells exposed to 15mM or 25mM glucose for 1 day did not see a change in MMP9 (15mM 25.65±12.83pg/ml and 25mM: 18.00±8.84pg/ml) from the 5mM CTL cells (17.77±11.91pg/ml). At Day 7 of increased glucose exposure, MMP9 was not significantly different in the 15mM cells (11.60±12.49pg/ml) or the 25mM cells (14.82±10.24pg/ml). Neither group was significantly different from CTL cells at Day 10 (15mM: 17.76±6.30pg/ml and 25mM: 7.15±11.00pg/ml).

TIMP2 levels in the membrane were significantly different between the different groups (p=0.0360; **Figure 6C**). After 1 day of exposure to increased 15mM glucose, cells saw a decrease in the levels of membrane TIMP2 (125.60±57.21pg/ml; p=0.0490) compared to the 5mM CTL cells (207.9±73.67pg/ml). No change was seen in the 25mM cells after 1 day (151.80±4306pg/ml). TIMP2 levels remained constant at Day 7 of exposure (15mM: 126.0±29.07pg/ml and 25mM: 149.9±60.01pg/ml), with 15mM remaining significantly lower than the CTL cells (p=0.0041). By Day 10, TIMP2 increased slightly in 15mM cells (142.10±36.81pg/ml) to levels that were no longer significantly different from CTL. However, the 25mM cells TIMP2 levels (241.40±84.54pg/ml) did no significantly differ from CTL cells.

Discussion

The purpose of this study was to investigate the mechanism of DPP-IV shedding from skeletal muscle membrane upon stimulation of the skeletal muscle. Additionally, this study aimed to determine the effects of hyperglycemia on this mechanism. The findings reveal that, while there were many changes in DPP-IV, MMP2, pro-MMP9, and TIMP2, there were no statistically significant changes with stimulation except for the significant decrease in pro-MMP9 when cells were treated with an MMP9 inhibitor. Alternatively, significant changes were seen when the cells were exposed to increased amounts of glucose for variable times. Results showed MMP2 is increased in 25mM glucose after 7 and 10 days of exposure, and TIMP2 is decreased after 1 and 7 days of exposure to 15mM glucose. Despite these changes, there were no significant differences in the DPP-IV shed or membrane bound DPP-IV following stimulation.

DPP-IV Released following Stimulation

Following stimulation, there was no change in the DPP-IV activity of the media. Additionally, there was no change in the amount of membrane DPP-IV, suggesting that any potential DPP-IV shed into the media was not enough to cause a significant loss of membrane bound DPP-IV. This agrees with what was found by Raschke et al, even though their time points for collecting media were at least 4 hours after stimulation occurred [15]. As demonstrated by this study and previous studies [15, 22], skeletal muscle cells actively shed DPP-IV without any known stimulus being required, making it difficult to determine any shedding caused by stimulation.

The findings of Koskinen and colleagues showed an increase in TIMP2 inhibition of MMP2 in peritendinous interstitial space following exercise [126]. This would suggest a potential change in these ECM proteins would be seen with stimulation of cells; however no changes were observed with stimulation of the cells. Previous studies also showed that immediately following exercise, MMP9 was significantly increased [99, 100, 127]. But there were no changes in pro-MMP9 upon stimulation of the cells. It is possible that there was not enough stimulus from the stimulation to cause any changes in MMP9.

Shedding of DPP-IV following Stimulation with MMP Inhibition

Interestingly, upon inhibition of MMP2 or MMP9, DPP-IV shed by the membrane following stimulation appeared to decrease; however analysis showed there were no statistically significant changes. Previous literature by Neidert et al. and Rohrborn et al. showed MMP2 and MMP9 play a role in the shedding of DPP-IV from skeletal muscle membrane [21, 22]. Additionally, there were no differences seen in the amount of membrane bound DPP-IV with MMP inhibition. This could suggest two possibilities. The first is that DPP-IV in the amount being shed from the membrane without inhibition is so small that it makes it nearly impossible to detect any changes with inhibition. The second is that DPP-IV can be quickly replenished on the surface of skeletal muscle cells once it is shed. In a previous study, cells treated with AICAR to mimic exercise conditions via activation of AMPK saw a significant increase in DPP-IV mRNA, indicating that it is likely that DPP-IV can quickly be replenished on the cell surface [22].

When looking at ECM proteins, they all responded similarly dependent upon whether MMP2 or MMP9 was inhibited. With the inhibition of MMP2, there were no alterations in the ECM proteins with stimulation. The response to the MMP2 inhibitor is interesting in that when MMP2 is inhibited, MMP9 should respond normally; however, that is not the case suggesting that MMP9 is dependent upon the activation of MMP2. In 1995, a study by Fridman and colleagues showed the activation of MMP9 was mediated by MMP2 [128], and this finding has been seen in ECM remodeling in several settings including cancer and CVD [129, 130]. Another interesting thing to note with MMP2 inhibition was the corresponding lack of change in TIMP2 with inhibition of MMP2.

ECM proteins were decreased from the CTL cells after inhibiting MMP9. While only pro-MMP9 was significantly lower, the decrease in MMP2 and TIMP2 both approached significance, indicating that there is a response in the ECM when MMP9 is inhibited. Because pro-MMP9 was decreased, this suggests that the activated form MMP9 was inhibited rather than pro-MMP9. Conversely to MMP2 inhibition, the response of MMP2 was similar to what was seen with CTL stimulation. However, there is still a decrease in the amount of DPP-IV being shed. This agrees with what was previously found suggesting that MMP9 plays a greater role in the shedding of DPP-IV from the membrane [21, 22].

DPP-IV Shedding following 15mM Hyperglycemia and Stimulation

After the cells were exposed to 15mM hyperglycemic conditions (mimicking pre-diabetic conditions), there were no significant changes seen in the shedding of DPP-IV upon stimulation

across the 3 different time points. Additionally, there were no changes in the membrane bound DPP-IV with the increased glucose exposure. This was not expected, as the hypothesis was that with increased glucose, there would be a moderately increased shedding of DPP-IV in the beginning stages of exposure leading to a corresponding decrease as exposure time increased.

The measured ECM proteins proved to have interesting responses to the glucose exposure. After 1 day of increased glucose, there were no changes in MMP2 or pro-MMP9, suggesting pre-diabetic levels of glucose do not have an acute effect on MMP2 or MMP9 levels. The opposite is true for TIMP2. Acute pre-diabetic glucose exposure brought a decrease in TIMP2. The decrease in TIMP2 agrees with a previous study showing a decrease in TIMP2 following glucose exposure in vascular cells [32]. The interesting thing is that an increase in pro-MMP9 was expected, but not seen. Death et al. showed increased MMP9 in macrophages with glucose exposure [31]. Additionally, MMP9 has a connection to inflammation [131], so it was possible that changes in the first 24 hours would have been related to acute inflammation brought on by the hyperglycemia. IL-6, which is known to influence glucose uptake [132], was shown to have altered actions in diabetic skeletal muscle [133] and may include a modulatory effect on MMP9 [99]. Altogether, the lack of shedding of DPP-IV from the membrane corresponds with the lack of change seen with the MMPs. However, why the decrease in TIMP2 did not cause a change in MMP2 remains unclear.

At Day 7, there were no changes in MMP2 or pro-MMP9 in the 15mM cells, and there was no change in TIMP2 from Day 1. TIMP2 remained significantly lower than CTL cells, indicating that it remains stable following the acute response. However, it is uncertain as to why

MMP2 did not change with TIMP2 levels remaining decreased as seen on Day 1 as Tayebjee et al. showed TIMP2 and MMP2 levels are related [107].

Alterations in ECM proteins at Day 10 of 15mM hyperglycemia saw an interesting response. MMP2 and pro-MMP9 remained at normal levels, while TIMP2 returned to values that were no longer significantly different from CTL cells. MMP9 overexpression is seen in Type 2 diabetics due to the effect of hyperglycemia on sirtuin1 (SIRT1). The oxidative stress-dependent SIRT1 has a negative effect on MMP9, such that when SIRT1 is decreased, there is an increase in both the activity and expression of MMP9 [134, 135]. However, these were diabetic conditions, leaving the possibility that the exposure to 15mM was not high enough to elicit a response.

DPP-IV Shedding following 25mM Hyperglycemia and Stimulation

Acute exposure to diabetic levels of hyperglycemia (25mM glucose) did not cause any changes in the DPP-IV shed from the cells or the membrane bound DPP-IV compared to CTL cells. This follows data from our lab that show plasma DPP-IV was not altered following maximal exercise preceded by fructose ingestion (unpublished results). Additionally, DPP-IV shedding and membrane DPP-IV was not altered following the 7 and 10 days of chronic exposure to 25mM glucose. Previous work from the Kluess lab showed that plasma DPP-IV is positively correlated with lean mass in healthy, normal glucose tolerant individuals [14], where other studies have shown increases in plasma DPP-IV with diabetes [6-8]. This would suggest

that the alteration of plasma DPP-IV in diabetics is not due to changes in the membrane bound DPP-IV of skeletal muscle.

MMP2, pro-MMP9, and TIMP2 did not experience any change from normal conditions after 1 day of 25mM glucose exposure. The response of MMP2 and pro-MMP9 match that seen with 15mM conditions, contrary to what was originally hypothesized, but matches the lack of DPP-IV shed from the membrane. However, TIMP2 was not significantly altered as seen in the acute exposure of pre-diabetic levels of hyperglycemia.

Seven days of diabetic levels of glucose exposure resulted in a change to only one of the measured ECM proteins. After chronic 25mM hyperglycemia, MMP2 is significantly increased. As there was no change seen with acute exposure, this suggests the time course for change in MMP2 might take longer than 24 hours. Ho et al. showed changes in MMP2 following 48 hours of glucose exposure [32]. TIMP2 and pro-MMP9 stay approximately where they were after 1 day. This is odd in that the interaction with MMP2 and TIMP2 does not agree, similar to what was seen with 15mM glucose. An alteration in MMP2 should correspond with a change in TIMP2 [32].

Finally, following 10 days of diabetic exposure, only MMP2 is significantly increased from the CTL cells agreeing with earlier studies. Cardenelli et al. found that in Type 2 diabetic smooth muscle cells, there is over activity of MMP9 due to the imbalance of TIMP3 associated with disrupted SIRT1 levels [135]. However, pro-MMP9 was not found to be different after 10 days of exposure to 25mM glucose. TIMP2 was not changed from normal levels which again asks the original question of what is the interaction between the increased MMP2 and TIMP2. It

could possibly be a compensatory mechanism by which more MMP2 is produced to allow for greater combat of inhibition by TIMP or it could be that more TIMP2 is produced as a response to increased MMP2 to assist in both inhibition and activation.

Future Directions

From here, a few points of interest develop that solicit further investigation. One is that in both pre-diabetic and diabetic hyperglycemic conditions, there is a reduction in membrane bound DPP-IV, despite the changes in ECM proteins. It is likely that there is an increased need for DPP-IV within the cytosol to combat the elevated glucose conditions, leaving less to be released by the membrane upon any stimulus, including stimulation. Future studies should investigate if intracellular pathways exist that can be altered by glucose and would employ DPP-IV

The second is skeletal muscle cells saw acute alterations in ECM proteins that either remained constant or varied in their response after chronic hyperglycemia. While some, did not change after 1 day of increased glucose, others did. It would be interesting to investigate the recovery of acute hyperglycemic bouts on skeletal muscle cells to see how the ECM proteins responded to varying amounts of glucose, similar to a Western diet, and if they elicited the same results as a consistent dose of increased glucose.

And finally, there were several times where the interaction between MMPs, TIMP2, and/or membrane bound DPP-IV did not agree with what was hypothesized. While MMP2 and MMP9 were identified as the two MMPs most likely to have an effect on skeletal muscle DPP-IV shedding, the effect of a complete protease inhibitor could possibly address whether or not

there are other mechanisms for the shedding of membrane bound DPP-IV and if this is altered by hyperglycemic conditions.

Limitations

The overarching limitation of this study was the use of cell culture, which may have made the scale of many measurements too small to draw definitive conclusions. For example, the measures of DPP-IV shed into the media were around 1U/L, leaving much variation in the measurements and large standard deviations. Additionally, the beginning time frame may have been too long to determine any changes in DPP-IV. When the pre levels of DPP-IV are compared, there is a marked decrease in DPP-IV in the media for pre-diabetic and diabetic conditions starting at 1 Day. This indicates that the responses may occur in a timeframe less than 24 hours. Another limitation to utilizing cell culture for this study was the use of electrical stimulation to mimic muscle contractions. While visible contractions of myotubes occurred, not all contracted. This along with the small scale made it difficult to conclusively determine whether or not DPP-IV was shed from the membrane with stimulation. And finally, there was a limit as to how many measures could be made based on the limited amount of membrane sample cell culture was able to yield. A study with a larger model such as a mouse or rat should be used to investigate the potential effects of muscle contractions on DPP-IV shedding to allow for greater scale of measures as well as increased sample yield for measurements to be made.

Conclusion

From this study, no significant changes in shedding of DPP-IV from skeletal muscle membrane were demonstrated following stimulation in normal, pre-diabetic, or diabetic conditions. Additionally, membrane DPP-IV and ECM proteins were not significantly altered with stimulation in normal cells. There were, however, significant changes seen in membrane DPP-IV and ECM proteins with pre-diabetic and diabetic conditions across the different time points, suggesting that glucose does in fact alter the different proteins involved in the DPP-IV shedding mechanism. While it is likely the cell culture model was too small to detect any significant changes in DPP-IV shedding with stimulation and glucose exposure, the current findings suggest that skeletal muscle DPP-IV and ECM proteins may be altered with stimulation. This attributes them to be possible modulators of functional sympatholysis, and with the onset of hyperglycemia, this mechanism can be considerably disrupted. Future research should investigate these findings in a larger model to determine if in fact DPP-IV shedding can occur with exercise and if it is affected by hyperglycemia. And because these proteins are involved in multiple actions such as satellite cell proliferation, glucose uptake, and inflammation in addition to the potential role in blood flow, the effect of hyperglycemia on the function of the skeletal muscle membrane carries even more importance.

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Figures

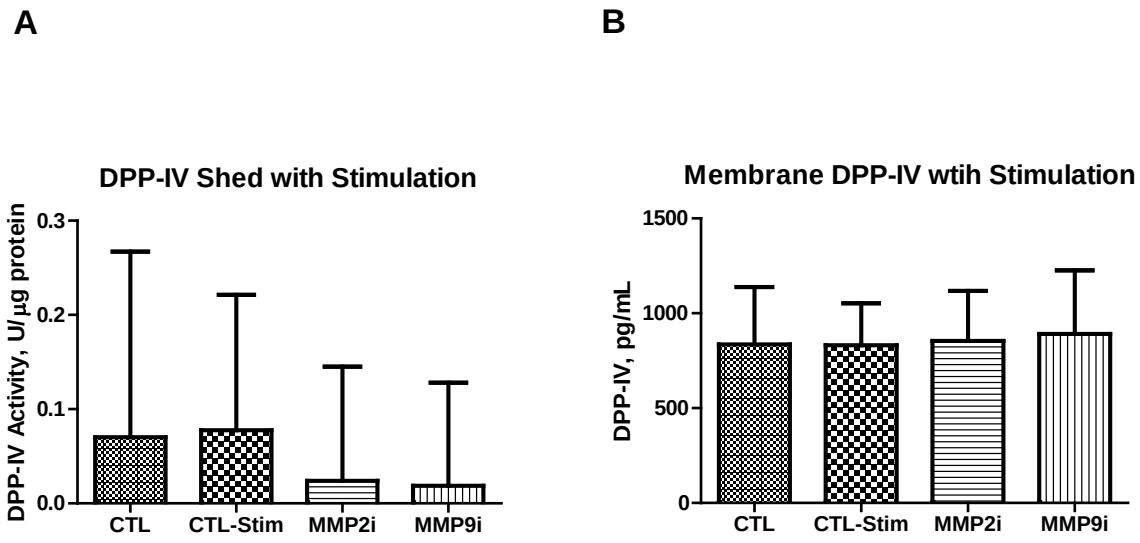

Figure 3.

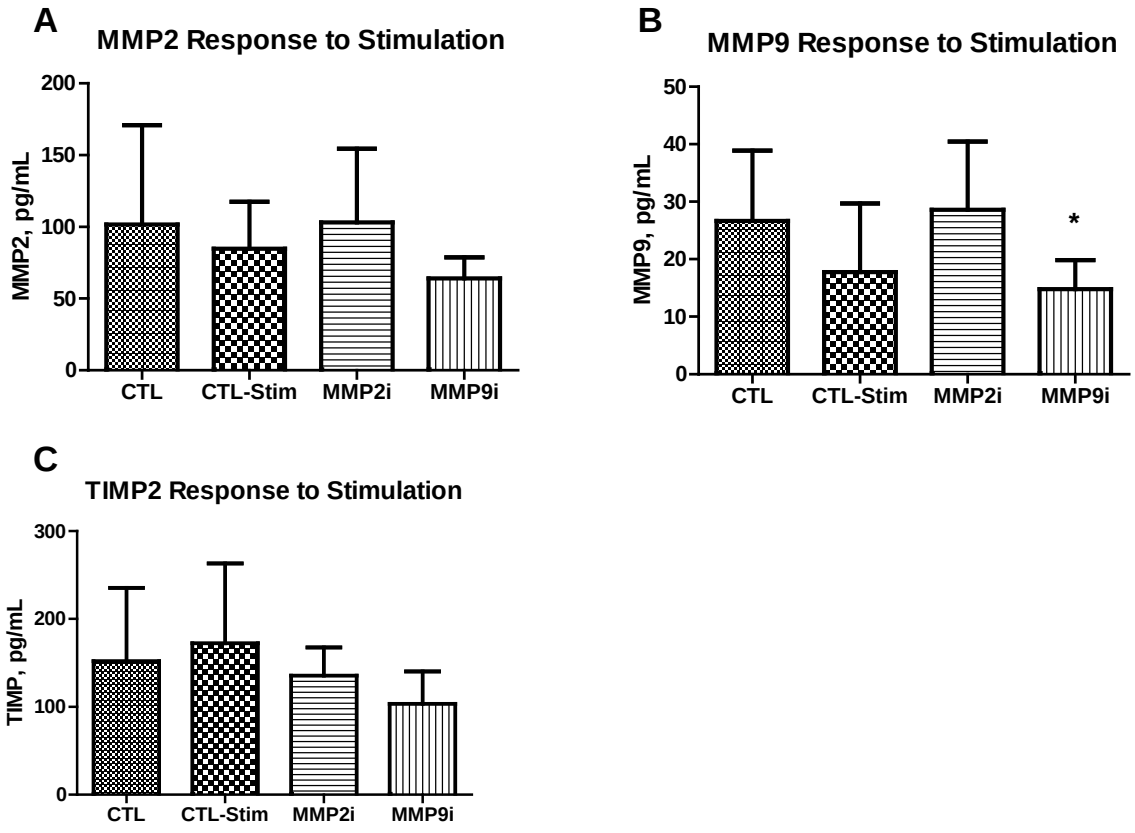
A
MMP2 Response to Stimulation
MMP2, pg/mL
200
150
100
50
0
CTL
CTL-Stim
MMP2i
MMP9i
B
MMP9 Response to Stimulation
MMP9, pg/mL
50
40
30
20
10
0
*
CTL
CTL-Stim
MMP2i
MMP9i
C
TIMP2 Response to Stimulation
TIMP, pg/mL
300
200
100
0
CTL
CTL-Stim
MMP2i
MMP9i

Figure 4.

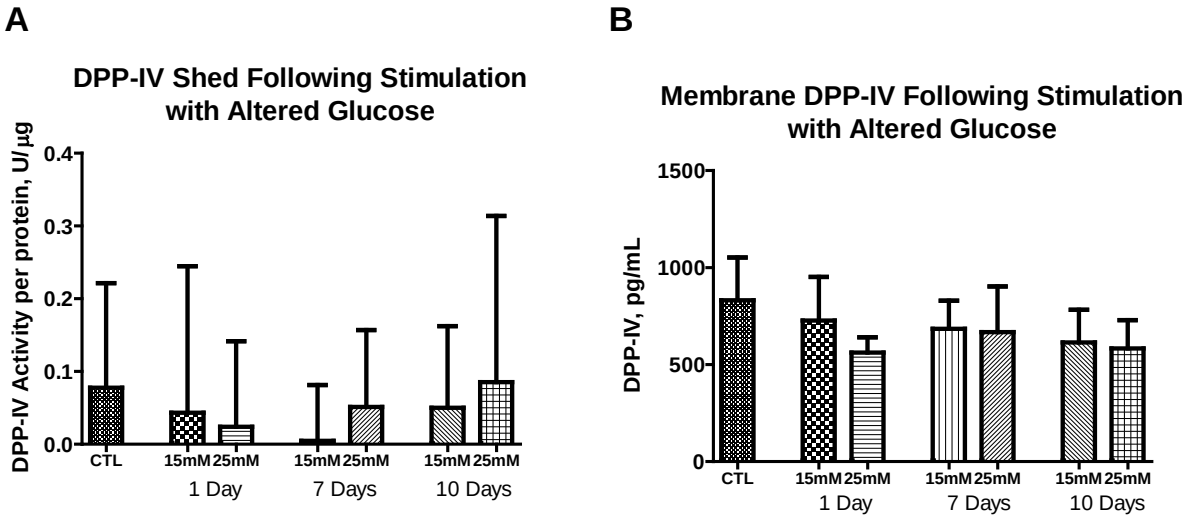
A
DPP-IV Shed Following Stimulation with Altered Glucose
DPP-IV Activity per protein, U/µg
0.4
0.3
0.2
0.1
0.0
CTL
15mM
25mM
15mM
25mM
15mM
25mM
1 Day
7 Days
10 Days
B
Membrane DPP-IV Following Stimulation with Altered Glucose
DPP-IV, pg/mL
1500
1000
500
0
CTL
15mM
25mM
15mM
25mM
15mM
25mM
1 Day
7 Days
10 Days

Figure 5.

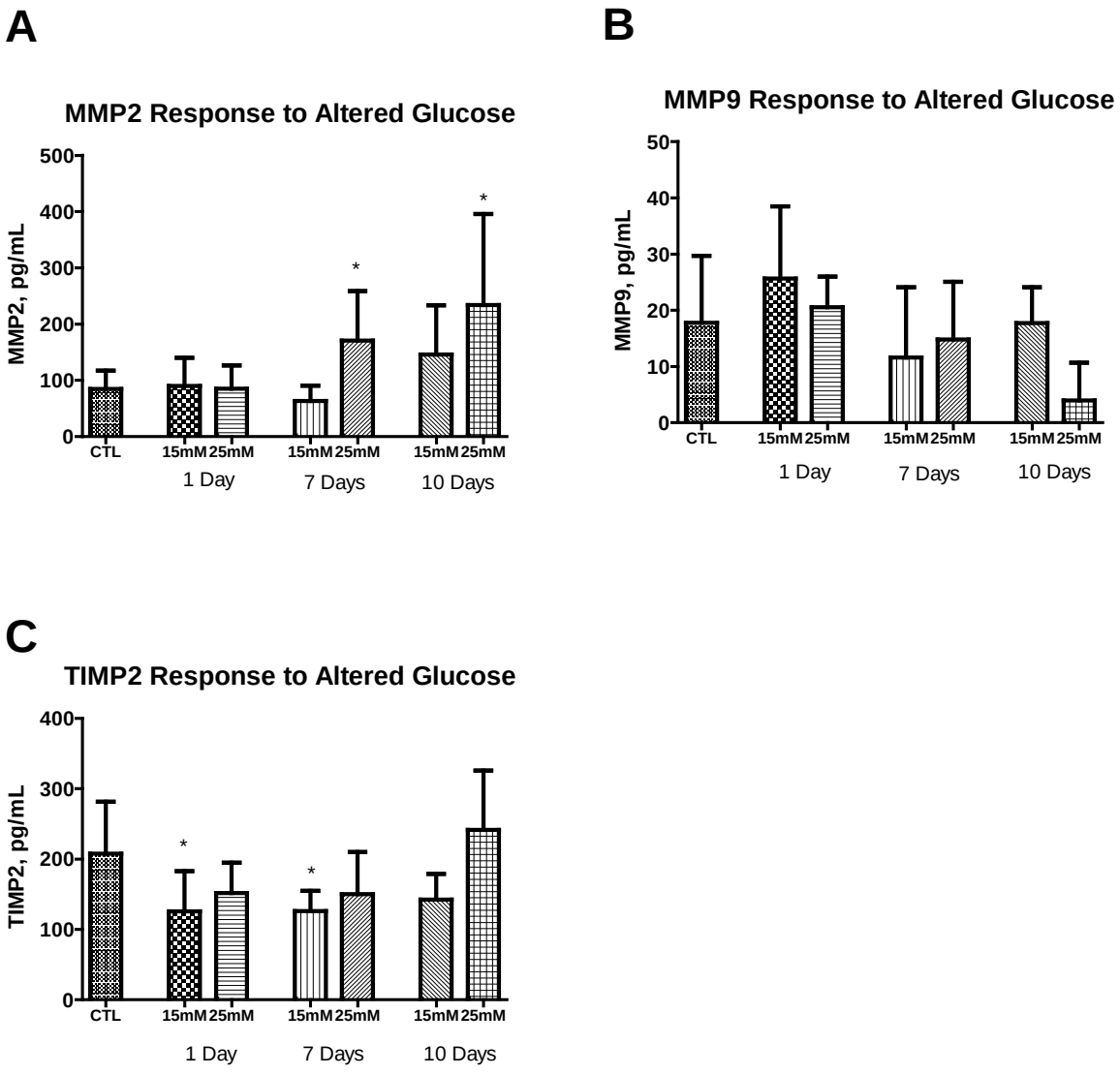
A
MMP2 Response to Altered Glucose
MMP2, pg/mL
500
400
300
200
100
0
*
*
CTL
15mM
25mM
15mM
25mM
15mM
25mM
1 Day
7 Days
10 Days
B
MMP9 Response to Altered Glucose
MMP9, pg/mL
50
40
30
20
10
0
CTL
15mM
25mM
15mM
25mM
15mM
25mM
1 Day
7 Days
10 Days
C
TIMP2 Response to Altered Glucose
TIMP2, pg/mL
400
300
200
100
0
*
*
CTL
15mM
25mM
15mM
25mM
15mM
25mM
1 Day
7 Days
10 Days

Figure 6.

Group	DPP-IV Shed	Membrane DPP-IV	MMP2	pro-MMP9	TIMP2
CTL	0.0702±0.20; 12	835.60±302.90; 8	101.50±69.37; 8	26.64±12.25; 8	152.20±83.19; 8
CTL-Stim	0.0776±0.14; 11	831.90±220.69; 8	84.74±32.73; 8	17.77±11.91; 8	172.4±90.63; 8
MMP2i	0.0240±0.12; 12	854.50±263.20; 8	103.10±51.22; 8	28.61±11.88; 8	135.50±32.09; 8
MMP9i	0.0185±0.11; 10	890.10±335.90; 8	64.00±14.71; 8	14.81±5.04; 8	103.30±37.15; 8

Table 1

Comparison	F-value	Degrees of Freedom	p Value
DPP-IV Shed	0.4724	3	0.7032
Membrane DPP-IV	0.0703	3	0.9753
MMP2	1.220	3	0.3209
pro-MMP9	3.136	3	0.0411
TIMP2	1.557	3	0.2219

Table 2

Group	DPP-IV Shed	Membrane DPP-IV	MMP2	pro-MMP9	TIMP2
CTL	0.0776±0.14;8	831.90±220.698	84.74±32.73; 8	17.77±11.91;8	207.94±73.64;6
15mM-1D	0.0433±0.20; 8	728.25±224.448	90.18±49.96; 8	25.65±12.83; 8	125.62±57.21; 8
15mM-7D	0.0046±0.08; 8	684.49±144.668	63.18±27.37; 8	11.60±12.49; 8	126.04±29.07; 8
15mM-10D	0.0787±0.07; 8	614.58±167.408	145.81±87.58;7	17.76±6.30; 8	142.15±36.81; 8
25mM-1D	0.0240±0.12; 8	562.37±78.64; 7	84.91±41.81; 8	20.57±5.43; 7	151.83±43.06; 8

25mM-7D	0.0511±0.11; 8	667.61±236.188	170.43±88.25; 8	14.82±10.23; 8	149.89±60.01; 8
25mM-10D	0.0854±0.29; 8	583.79±146.268	233.71±161.96; 7	7.15±11.00; 8	241.39±84.54; 6

Table 3

Comparison	F- or H-value	Degrees of Freedom	p Value
DPP-IV Shed	0.445	6	0.8460
Membrane DPP-IV	2.001	6	0.0840
MMP2	16.40*	6	0.0120
pro-MMP9	2.617	6	0.0280
TIMP2	13.46*	6	0.0360

Table 4

Figure Legends

Figure 1. DPP-IV Interaction with GLP-1. Post-prandial GLP-1 is released from the gut, with the purpose of signaling the pancreas to release insulin. However, DPP-IV cleaves GLP-1 rendering it inactive and unable to signal for insulin release.

Figure 2. Role of DPP-IV in NPY-Mediated Vascular Response. NPY_{1-36} when left in its full length form acts on Y1 receptors to mediate vasoconstriction and modulation of vascular tone. However, DPP-IV can be shed from skeletal muscle by MMPS and act on NPY_{1-36} to shorten it to NPY_{3-36}. This modulation changes its affinity from Y1 to Y2 receptors, allowing relaxation of the vasculature through vasodilation.

Figure 3. DPP-IV Response to Stimulation Panel. A) The shedding of DPP-IV was measured by the collection of pre and post stimulation media samples and measuring for DPP-IV activity per protein. No significant changes were seen in the delta values for CTL-Stim, MMP2i, and MMP9i when compared to CTL. B) Membrane cell fractions were measured for DPP-IV protein concentrations. No significant changes were seen in CTL-Stim, MMP2i, and MMP9i cells compared to CTL.

Figure 4. ECM Protein Responses to Stimulation Panel. A) Membrane cell fractions were measured for MMP2 protein concentrations. No significant changes were seen in CTL-Stim, MMP2i, and MMP9i cells compared to CTL. B) Membrane cell fractions were measured for pro-MMP9 protein concentrations. Levels of pro-MMP9 in MMP9i cells were significantly lower than CTL cells, but no significant changes were seen in CTL-Stim and MMP2i compared to CTL. * indicates significantly different from CTL. C) Membrane cell fractions were measured for TIMP2 protein concentrations. No significant changes were seen in CTL-Stim, MMP2i, and MMP9i cells compared to CTL.

Figure 5. DPP-IV Response to Altered Glucose Panel. A) The shedding of DPP-IV was measured by the collection of pre and post stimulation media samples and measuring for DPP-IV activity per protein. No significant changes were seen in the delta values for any of the cells when compared to CTL. B) Membrane cell fractions were measured for DPP-IV protein concentrations. No significant changes were seen in the 15mM cells 25mM cells when compared to CTL. * indicates significantly different from CTL.

Figure 6. ECM Protein Responses to Altered Glucose Panel. A) Membrane cell fractions were measured for MMP2 protein concentrations. There were no significant differences in 15mM cells from CTL cells. 25mM cells were significantly different from CTL at 7 and 10 days, but not 1 Day. * indicates significantly different from CTL B) Membrane cell fractions were measured for pro-MMP9 protein concentrations. No significant changes were seen in any of the cells exposed to hyperglycemic conditions at any of the time points compared to CTL cells. *

indicates significantly different from CTL C) Membrane cell fractions were measured for TIMP2 protein concentrations. 15mM cells were significantly decreased at Days 1 and 7 compared to CTL cells, but not at 10 Days. No significant changes were seen in cells exposed to 25mM cells at the 3 time points. * indicates significantly different from CTL.

Table 1. Part 1 Group Statistics. Values are reported as mean ± SD and the n for each group.

Table 2. Part 1 Analysis. The F-values, degrees of freedom, and p value for each analysis is reported

Table 3. Part 2 Group Statistics. Values are reported as mean ± SD and the n for each group.

Table 4. Part 2 Analysis. The F-values (*H values if non-parametric test), degrees of freedom, and p value for each analysis is reported