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# The Effects of Type 1 Diabetes on the Mechanoreflex in Rats

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# The Effects of Type 1 Diabetes on the Mechanoreflex in Rats

**by**

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# Dedication

This thesis is dedicated to my parents, Jesus and Maria Ana. Thank you for always supporting me and showing me unconditional love. I am eternally grateful to you both for encouraging me to stay true to myself and follow my dreams. I don't know where I would be without you.

Esta tesis está dedicada a mis padres, Jesús y María Ana. Gracias por siempre apoyarme y mostrarme amor incondicional. Estoy eternamente agradecida a ambos por alentarme a mantenerme fiel a mí misma y seguir mis sueños. No sé dónde estaría sin ustedes.

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# Abstract

## The Effects of Type 1 Diabetes on the Mechanoreflex in Rats

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Mechanical allodynia is present as early as four days in streptozotocin (STZ) induced type 1 diabetes mellitus (T1DM) in rats. This is thought to occur through mechanisms affecting the same thin fiber afferents that evoke the mechanoreflex. In this study, the purpose was to determine the effects of T1DM on the mechanoreflex. We injected (i.p.) 50 mg/kg of Streptozotocin (STZ) or the vehicle (CTL) in both sexes and waited 1wk (STZ: BW=251.2±11.47g, glucose=468.1±23.18mg/dL, HbA1C=6.09±02%; CTL: BW=351.5±21.01g, glucose=176.3±10.94mg/dL, HbA1C=4.23±0.12%). In unanesthetized decerebrate rats, we stretched the Achilles tendon for 30s and measured the pressor and cardioaccelerator responses. We then compared the pressor and cardioaccelerator responses to tendon stretch before and after inhibition of Piezo 1 and 2 channels. Inhibition was done by injecting GsMTx-4 (10μg/100μl), a selective mechano-gated Piezo channel inhibitor, into the arterial supply of the hindlimb muscles in rats. We found that the pressor (STZ: ΔMAP=42.1±8.3 mmHg, n=9; CTL: ΔMAP=18.7±4.0 mmHg, n=6; $p<0.05$) but not the cardioaccelerator (STZ: ΔHR=13.6±3.7 bpm, n=9; CTL: ΔHR=9.7±2.7 bpm, n=6; $p>0.05$) responses to tendon stretch were exaggerated 1wk after

injecting STZ. Furthermore, GsMTx-4 appears to attenuate the pressor (before GsMTx-4: $\Delta$MAP=65.33±4.98 mmHg; after GsMTx-4: $\Delta$MAP=46±4.16 mmHg, n=3; $p<0.05$) but not cardioaccelerator (before GsMTx-4: $\Delta$HR=16.67±4.18 bpm; after GsMTx-4: $\Delta$HR=13.33±6.77 bpm, n=3; $p>0.05$) responses to tendon stretch in rats 1wk after STZ injection. The developed tensions from tendon stretch were similar within each comparison. We conclude that the mechanoreflex is augmented in the early stage of T1DM and that Piezo channels likely play a role in this response.

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# Chapter 1: Literature Review

## INTRODUCTION

A propagation of physiological changes occurs at the onset of exercise. Heart rate, myocardial contractility, and blood pressure are increased to meet the increasing metabolic demand of exercising skeletal muscle (O'Leary et al., 1999; Amann et al., 2010). These changes are due to the withdrawal of parasympathetic output and increase in sympathetic output to the heart, blood vessels, and visceral organs. The two mechanisms responsible for these changes are the feedforward mechanism termed central command and the feedback mechanism termed the exercise pressor reflex. The exercise pressor reflex originates in exercising musculature and is made up of thinly myelinated group III and unmyelinated group IV afferents. These afferents create reflexive changes when stimulated by mechanical and metabolic stimuli produced from contracting skeletal muscle. This activity travels through dorsal roots and integrates at multiple sites in the brainstem that synapse onto autonomic preganglionic neurons (Kaufman & Forster, 1996).

In healthy populations, the exercise pressor reflex is necessary to redistribute blood flow and meet the demand from exercise. Contrary to the responses seen in healthy people, diseased states have shown alterations to these responses that may predispose these populations to cerebral and/or cardiovascular events. Type 1 Diabetes Mellitus (T1DM) is of great concern because the effects of the disease on the exercise pressor reflex are not well understood. In T1DM patients, cardiovascular disease presents as a major concern because oxidative stress and perturbations to the vascular endothelium appear in early stages of the disease (Elhadd et al., 1999). In contrast, high frequency of high intensity exercise is associated with reductions of cardiovascular events in this patient population (Tikkanen-Dolenc et al., 2017). Furthermore, in diabetic patients, the development of

peripheral neuropathy is thought to occur through the same afferents which evoke the exercise pressor reflex (Sun et al., 2012). Diabetic patients commonly experience the development of mechanical allodynia, which is a symptom of peripheral neuropathy in which painful sensation is caused by innocuous stimuli. In STZ-induced diabetic rats, the development of mechanical allodynia occurred as early as 5 days after injection (Sun et al., 2012). As it is a possibility that these afferents are altered, it is important to understand how the responses to exercise are affected to avoid the development of exercise intolerance.

The review will give a brief background on both the exercise pressor reflex and central command and will outline human and animal studies that have made pivotal findings of the exercise pressor reflex. Additionally, descriptive properties of stimuli arising from mechanical and metabolic components, specifically the thin fiber afferents that evoke the reflex will be discussed. Finally, an overview of the effects of diseased states on the reflexive alterations to circulation during exercise will be brought upon. Ultimately, the goal of this review is to highlight the importance of understanding the exercise pressor reflex in healthy and diseased populations because exercise is essential for prevention and treatment of diabetes.

## BACKGROUND

The early studies of Alam and Smirk highlighted several aspects of the circulatory control of the autonomic nervous system during exercise (Alam & Smirk, 1937). Prior to these studies, the general finding was that exercise increases systolic and diastolic pressure. The discovery made by Alam and Smirk was that the rise in blood pressure was sustained when the circulation was occluded even though exercise had ceased. This rise in blood pressure was dependent on the length of occlusion and the amount of work being done but

not on the production of pain (Alam & Smirk, 1937). These findings in man propagated further research on the topic as it became clear that the presence of a reflex was responsible for these changes, namely the exercise pressor reflex. This became evident since the occlusion of circulation allowed the sensory afferents to be the sole connection from the exercising muscle (Alam & Smirk, 1937). The exercise pressor reflex is a feedback mechanism that originates in contracting skeletal muscle to increase the supply of oxygenated blood and meet the increasing demand. The reflex increases the sympathetic nerve activity in resting muscles, which creates vasoconstriction, and allows for redistribution of the blood flow (Mark et at., 1985; Cui et al., 2006).

A competing process of the neural control of circulation during exercise is the feedforward mechanism known as central command. Central command was initially thought to originate in cortex centers that control motor systems in conjunction with respiratory and circulatory systems (Krogh & Lindhard, 1913). Studies in decorticate cats have shown that central command arises from hypothalamic locomotor regions and the primary adjustments during exercise do not necessarily come from a reflex (Eldridge et al., 1981; Eldridge et al., 1985). Furthermore, excitation of an inhibitory reflex of the motor neurons innervating the contracting muscle showed a greater contribution from central command, whereas reflex excitation of the same motor neurons showed a withdrawal from central command, while producing similar tensions (Goodwin et al., 1972). The key finding from this study was that the increased contribution of central command, brought on by inhibition, elicited a greater blood pressure response than when the contraction was not being inhibited. In contrast, when the motor neurons were aided by reflex excitation, which reduced the contribution from central command, the blood pressure response was less when compared to a normal contraction. These observations provided evidence for the

"irradiation" theory first postulated by Krogh and Lindhard. Additionally, they established that there is a mixed contribution from both central command and the exercise pressor reflex to the circulatory changes during exercise. T4he focus of this review will be on the exercise pressor reflex more so than central command. However, it is important to note that both central command and the exercise pressor reflex contribute to the hemodynamic changes that occur during exercise.

## HUMAN STUDIES

The Alam & Smirk studies provided the first evidence of a reflexive increase in blood pressure in humans. The subjects performed a handgrip exercise while their upper arm was occluded and blood pressure increased immediately. Even after the exercise ceases, blood pressure continued to increase. Furthermore, the duration of circulatory arrest was directly correlated with the increase in blood pressure (Alam & Smirk, 1937). These experiments suggested that the metabolites accumulated from contracting skeletal muscle were acting locally creating a reflexive response, now known as the metaboreflex. Since the circulation was occluded and blood could not leave the limb, they concluded that the metabolites were acting on sensory nerve endings.

In humans, it has been shown that during upright exercise the control of heart rate is mediated by increases in sympathetic activity and withdrawal of parasympathetic activity; the sympathetic stimulation becoming progressively more important as the workload of exercise increases (Robinson et al., 1966). These studies established the recruitment of the efferent pathways that control heart rate by blockade of either sympathetic, parasympathetic, or double blockade. The extent and selectiveness of the

blockade is a limitation that arises from this. Nonetheless, increases in sympathetic nerve activity in humans have been recorded during exercise (Mark et al., 1985; Herr et al., 1999; Cui et al. 2006). These studies have been done by recording muscle sympathetic nerve activity of non-exercising musculature while other muscles were exercising; a technique called microneurography. Although there are implications with microneurography in human studies, increases in sympathetic nerve activity to the resting muscles, as well as visceral organs, have been measured in animal models and have shown similar findings (Hayes & Kaufman, 2002; Kim et al., 2006; Koba et al., 2007). Furthermore, increases in muscle sympathetic nerve activity, mean arterial pressure, and heart rate have been measured in humans during passive stretch and voluntary contraction (Cui et al., 2006). These studies determined that while solely stimulating mechanoreceptors, the same sympathetic responses and reflexive changes are seen providing evidence that the mechanoreflex stimulates sympathetic responses in humans. Moreover, muscle sympathetic nerve activity has been shown to progressively increase during a series of bouts of contraction-rest cycles of the quadriceps muscle (Herr et al., 1999). As exercise progresses in the manner of contraction-rest cycles, there is an accumulation of metabolites that sensitizes the metaboreflex in humans; as a result, both the mechanoreflex and the metaboreflex play a role in increasing sympathetic nerve activity. Research on the metaboreflex in humans may be challenging because administration of inhibitors presents a myriad of effects when given systemically; when the inhibition is administered locally to the exercising muscle, there is a greater possibility of identifying precise mechanisms. Local inhibition of prostaglandins in the exercising muscle showed a reduction in muscle sympathetic nerve activity (Cui et al., 2007). This provides evidence that metabolites produced during exercise in humans plays a role in the exercise pressor reflex.

## ANIMAL STUDIES

The hypothesis derived from Alam and Smirk was supported when contraction of the muscles using ventral root stimulation elicited a pressor response in cats; that same response was abolished when the dorsal roots were severed proving that the reflexive alterations were carried through sensory pathways (Coote et al., 1971). Furthermore, the use of an anodal block, which uses a direct current to block large diameter myelinated fibers such as Group I and Group II afferents, showed no changes in the ventilatory and pressor responses to ventral root stimulation. On the other hand, a local anesthetic block, which when applied directly to dorsal roots blocks small diameter myelinated and unmyelinated fibers such as Group III and Group IV afferents before Group I and II, abolished the pressor and ventilatory responses normally seen with ventral root stimulation (McCloskey & Mitchell, 1972). This study determined that Group I and II afferents were not responsible for the reflexive changes that occur with exercise.

Research on animal models has made it possible to further explore the mechanisms of the exercise pressor reflex. Moreover, specific properties of the group III and IV afferents have been identified using animal models. The pattern of Group III and Group IV afferent discharge differed during static contraction and injection of bradykinin and capsaicin. At the onset of contraction Group III fibers discharged immediately and tapered off as contraction continued. Whereas, Group IV fibers discharged in later stages of the contraction. Injection of bradykinin stimulated equal number of Group III and IV fibers, while capsaicin stimulated significantly more Group IV than Group III fibers (Kaufman et al., 1983). This supported the notion that Group III afferents respond to both metabolic and mechanical stimuli, whereas Group IV afferents solely respond to metabolites that build up during exercise due to their delayed discharge.

During exercise sympathetic nerve activity increases to redistribute the blood away from the viscera and resting muscles to the exercising musculature. In decerebrate unanesthetized cats, it was determined that direct stimulation of both central command and the exercise pressor reflex increased renal sympathetic nerve activity. Both mechanism were also responsible for discharging different renal postganglionic sympathetic efferents (Hayes & Kaufman, 2002). Renal sympathetic nerve activity plays an important role in distributing blood throughout the body during exercise. During simulated isometric exercise in dogs, there were significant increases in hemodynamic properties, such as mean arterial pressure, heart rate, and cardiac output. There were also reductions in blood flow to the kidneys (Crayton et al., 1979). These changes were eliminated when dorsal roots were sectioned, suggesting that the exercise pressor reflex played a role in increasing hemodynamic properties. Stretch and repetitive contractions of hindlimb muscles in rats showed greater responses from renal sympathetic nerve activity in comparison to lumbar sympathetic nerve activity, which innervate hindlimb blood vessels; both RSNA and LSNA were enhanced when circulatory occlusion was present during contraction (Koba et al., 2007). These findings further support the idea that the exercise pressor reflex creates differential sympathetic outflow and this outflow is enhanced as muscle metabolites are produced during exercise. Furthermore, inhibition of mechanosensitive thin-fiber afferents showed a diminished renal sympathetic nerve response to static contraction and stretch (Kim et al., 2006). This suggested that the mechanoreflex plays a role in renal vasoconstriction.

**DISCHARGE PROPERTIES**

The exercise pressor reflex is made up of thinly myelinated and unmyelinated sensory afferents. These are a part of the mechano- and metaboreflex. Reflexive changes to heart rate, blood pressure, and ventilation have been recorded during static contraction (McCloskey & Mitchell, 1972; Mitchell et al., 1977), intermittent contraction (Kaufman et al., 1984), and stretch of the muscle (Cui et al., 2006). Studies like these have led to the identification of specific properties that differentiate between the sensory afferents that evoke the exercise pressor reflex.

Group III afferents, also known as A-δ fibers, have polymodal discharge properties and are stimulated by mechanical deformation and metabolite accumulation during exercise (Kumazawa & Mizumura, 1977; Kaufman et al., 1983). Group III afferents are thinly myelinated with a diameter of 1 – 6 μm and a general conduction velocity of 2.6 – 30 m/s (Mitchell & Schmidt, 1982). Conduction velocities reported in cats and dogs are between 2.5 and 30 m/s (McCord & Kaufman, 2010; Stone & Kaufman, 2015) and between 1.6-10 m/s in rats (Stone & Kaufman, 2015). These terminate as free nerve endings in skeletal muscles and other tissues. The most prominent distinction between Group III and IV afferents is their responsiveness to mechanical stimulation; for that reason, they are commonly termed mechanoreceptors. Group III afferents have been shown to discharge at the onset of contraction (Kaufman et al., 1983) and during localized pressure and stretch (Abrahams et al., 1984; Kaufman & Rybicki, 1987); therefore, they contribute to the mechanoreflex. In addition to their responsiveness to mechanical stimuli, Group III afferents have been shown to be stimulated by bradykinin (Kaufman et al., 1983), lactic acid (Rotto & Kaufman, 1988), arachidonic acid (Rotto & Kaufman, 1988), potassium (Kaufman & Rybicki, 1987), and a thromboxane $A_2$ mimetic (Kenagy et al., 1996). Of

these, lactic acid is a powerful stimulus of Group III afferents as they discharge almost immediately after injection (Rotto & Kaufman, 1988).

Group IV afferents, also known as C-fibers, are unmyelinated and terminate in free nerve endings of skeletal muscle and other tissues. Group IV afferents are the smallest and slowest of the sensory afferents with a diameter of ~1 μm and general conduction velocity less than 2.5 m/s (Mitchell & Schmidt, 1982). Conduction velocities in cats and dogs have been reported to be less than 2.5 m/s (McCord & Kaufman, 2010; Stone & Kaufman, 2015) and less than 1.6 m/s in rats (Stone & Kaufman, 2015). Different from Group III afferents, Group IV afferents have a delayed discharge between 5 – 30 seconds from the onset of contraction (Kaufman et al., 1983). Additionally, Group IV afferents have shown a greater response during ischemic contraction compared to a freely perfused contraction (Kaufman & Rybicki, 1987). Group IV afferents are predominantly stimulated by metabolites. In fact, bradykinin (Kaufman et al., 1983), potassium (Kaufman & Rybicki, 1987), lactic acid (Rotto & Kaufman, 1988), and arachidonic acid (Rotto & Kaufman, 1988) have been shown to stimulate Group IV afferents. Moreover, injection of ATP and α,β-methylene ATP, an analog of ATP, have been shown to stimulate predominantly Group IV afferents as well as slow conducting Group III afferents (Hanna & Kaufman, 2003; Reinöhl et al., 2003). Likewise, injection of prostaglandin E2 has also been shown to sensitize Group IV afferents (Mense, 1981). Group IV afferents are commonly termed metaboreceptors.

The metabolites that have been shown to stimulate the sensory arm of the exercise pressor reflex stimulate specific receptors that have been identified on these afferents. ATP has been shown to stimulate P2X3 receptors (McCord et al., 2010), prostaglandin E2 stimulates EP4 receptors (Southall & Vasko, 2001), and lactic acid stimulates acid-sensing

ion channel 3 (Molliver et al., 2005). Of relevance, these receptors all have been identified to play a role in nociception. Contrary to metabolically sensitive afferents, specific receptors for mechanically sensitive afferents are not well known. Recently, Piezo proteins, which make up Piezo 1 and 2 channels, have been shown to evoke the exercise pressor reflex (Copp et al., 2016a).

## SUMMARY AND SIGNIFICANCE

Exercise has long been prescribed for the management and prevention of metabolic diseases. The influence the exercise pressor reflex has on cardiovascular regulation during exercise makes it imperative to understand how disease alters the reflex. In a dilated cardiomyopathy model, rats showed an augmented exercise pressor reflex in comparison to their healthy counterparts (Smith et al., 2003). However, exercise training in coronary heart failure blunted the occurrence of an exaggerated exercise pressor reflex that normally develops when exercise training is not present (Wang et al., 2010). Other diseases that have shown similar alterations to the reflex include hypertension (Leal et al., 2008; Koba et al., 2012; Mizuno et al., 2016) and peripheral artery disease (Stone & Kaufman, 2015; Copp et al., 2016b).

As of 2014 diabetes mellitus in an adult population, including Type I and II, had a prevalence of 8.5% (WHO, 2016). In the United States, 29.1 million people have diabetes and the estimated costs exceed $200 billion (CDC, 2014). T1DM is a form of diabetes in which pancreatic beta cells are destroyed due to or precipitated by an autoimmune response and creates deficiency in insulin production (WHO, 1999). The lack of insulin production, without the appropriate management, creates a hyperglycemic state, which can impair body

systems. In fact, there is a 2 to 3-fold greater risk of stroke and myocardial infarctions in people with diabetes (The Emerging Risk Factors Collaboration, 2010). Additionally, in diabetics the underlying cause of death that most commonly occurred was cardiovascular disease, constituting for 44% of deaths in T1DM (Morrish et al., 2001). Contrary to that, high frequency and high intensity exercise has been shown to reduce the risk of cardiovascular events in type 1 diabetics (Tikkanen-Dolenc et al., 2017).

Diabetic peripheral neuropathy, synonymously known as distal symmetric polyneuropathy, is present in at least 20% of people with T1DM (Pop-Busui et al., 2017). Symptoms of neuropathy include numbness, burning, prickling paresthesia, dysesthesias, and allodynia; these beginning distally in the feet (England et al., 2005). It is believed that sensory afferents play an important role in the development of allodynia. Specifically, smaller diameter fibers such as Aδ-fibers and C-fibers are of interest. In STZ induced diabetic rats, mechanical allodynia presented early on after injection and this neuropathic pain may be caused in part by an enhanced P2X3 receptor activity and increased membrane expression (Xu et al., 2011). However, Aδ-fibers have been reported to have lower activation thresholds and augmented responses to Von Frey filaments in comparison to the metabosensitive C-fibers in rats with allodynia present (Khan et al., 2002). Additionally, inhibition of the mechanoreflex in a simulated model of peripheral artery disease in rats has been show to attenuate the augmented pressor response (Copp et al., 2016b). This makes the mechanoreflex of primary interest in other diseases.

Since the development of peripheral neuropathy is thought to affect the same afferents that evoke the exercise pressor reflex, it is of importance to understand how diabetes affects the responses to exercise. The purpose of this study was to 1) determine

the effects of T1DM on the mechanoreflex in rats and 2) determine the role of mechanically sensitive Piezo 1 and 2 channels on the mechanoreflex in T1DM rats. The following hypotheses were tested: 1) STZ-induced diabetes will exaggerate the mechanoreflex in rats and 2) mechanically sensitive Piezo 1 and 2 channels found on the afferent endings of the skeletal muscle play a role in evoking the mechanoreflex in STZ rats.

# Chapter 2: Methods

## ETHICAL APPROVAL

Experiments were performed on adult Sprague-Dawley rats of either sex (n=15; Taconic Biosciences, Hudson, NY, USA). Descriptive characteristics can be seen in Table 1. Rats were housed at the Animal Resources Center (ARC) of the university, which provided food and water *ad libitum* and maintained the rats on a 12-h light/dark cycle. All procedures and protocols were approved and in compliance with the ethical guidelines of the Institutional Animal Care and Use Committee of the University of Texas at Austin.

## DIABETES INDUCTION

Rats were randomly assigned to experimental groups. To induce Type 1 Diabetes Mellitus (T1DM), anesthesia was initialized with a mixture of 5% isoflurane-95% oxygen. Sedation was maintained by placing the rat on a nose cone with a mixture of 3% isoflurane-97% oxygen. Body weight of the rat was measured using a digital compact scale (CS 2000, Ohaus Corporation, Parsippany, NJ). Streptozotocin (STZ; Sigma Aldrich, St. Louis, MO) dosage was determined by taking 50 mg/kg of bodyweight and then mixed in citrate buffer. Prior to injection, baseline blood glucose (Stat Strip Xpress, Nova Biomedical, Waltham, MA) and Hemoglobin A1c (HbA1c; A1C Now$^{+}$ Multi-Test HbA1c System, PTS Diagnostics designs, Indianapolis, Indiana), measurements were taken using venous blood samples taken from the tail. An intraperitoneal injection of 0.2 mL of STZ mixture was injected at the lower left quadrant. Control rats were taken through the same procedure but were injected with 0.2 mL of citrate buffer (Sigma Aldrich, St. Louis, MO). After injection, the rats were injected subcutaneously with lactated ringers to prevent dehydration as STZ injection increased their urine output. Rats were transported back to the ARC after they

regained consciousness and returned to normal behavior. Diabetes was determined by blood glucose levels greater than 300mg/dL and HbA1c levels greater than 6.5%.

### SURGICAL PROCEDURES

On the day of experiment, rats were initially anesthetized with a mixture of 5% isoflurane-95% oxygen inhalation for 5 minutes. Anesthesia was then sustained with a mixture of 3% isoflurane-97% oxygen on the nose cone. During this time, the rat was shaved and venous blood samples were taken from the tail to obtain blood glucose and HbA1c measurements. The trachea was cannulated and the rat was mechanically ventilated with a mixture of 2% isoflurane-98% oxygen. The right jugular vein was cannulated with a PE-50 catheter for delivery of drugs and fluids. Both common carotid arteries were cannulated with a PE-50 catheter for measurement of blood pressure and heart rate. The carotid catheters were also used to obtain arterial blood samples to measure arterial blood gases and pH using an automated blood gas analyzer (Nova Biomedical, Waltham, MA). The left common iliac artery and vein were isolated from connective tissue and a suture was placed around the vessels to create a snare. When the snare was tightened, the circulation of the leg was blocked sequentially trapping the injectate. The left superficial epigastric artery, which branches from the femoral artery, was cannulated with a PE-8 catheter for local drug injection of the hindlimb. Prior to decerebration, 0.2 mL of Dexamethasone (2mg/mL; Aspen Veterinary Resources, Greeley, CO) were injected intravenously to reduce brain edema. The rat was placed into a Kopf stereotaxic frame to perform a precollicular decerebration. During decerebration, rectal temperature was monitored and maintained between 37-38°C with an isothermal heating pad and lamp. After removal of the skin and skull, all neural tissue rostral to the incision was aspirated.

Anesthesia was terminated following decerebration and the rat was mechanically ventilated with room air. There was at least 60 minutes before the beginning of the experimental protocol to allow blood pressure to stabilize. The biceps femoris of the left leg was separated from the triceps surae. Surrounding connective tissue was removed from the triceps surae and the Achilles tendon was detached at the calcaneal bone. The Achilles tendon was tied with a string that was connected to a force-displacement transducer (FT-03, Grass Instruments, West Warwick, RI) for measurement of tension produced using a rack and pinion. The body of the rat was set into a spinal unit with a vertebral clamp at the thoracic spine and hip spikes at the ilium of the pelvis. The left hindlimb was clamped at the ankle joint and the knee was stabilized so it would not externally rotate during the stretch maneuver. At the end of the experimental protocols, arterial blood samples were collected and a laminectomy from L3-S1 was performed to expose the spinal cord and roots. Dorsal root ganglion neurons at L4 and L5 were removed and frozen in HBSS buffer (Sigma Aldrich, St. Louis, MO) at -80°C until processed.

**EXPERIMENTAL PROTOCOLS**

After surgical preparation, a carotid catheter was attached to a fluid-filled WE DTX-1 BP transducer and BPM-832 Amplifier for measurement of blood pressure, pulse pressure, and tension. These were monitored continuously using Spike2 acquisition system (Cambridge Electronic Design, Cambridge, UK) and heart rate was calculated beat to beat using the pulse pressure. Arterial blood gases and pH were maintained within normal limits by adjusting ventilation, $O_2$ supply, and intravenous injection of sodium bicarbonate (8.5%). At the beginning of the experiment, rats were paralyzed with 0.5 mL of pancuronium bromide (Sigma Aldrich, St. Louis, MO) injected intravenously. Continuous

doses were given every 30 minutes to ensure paralysis was maintained. The triceps surae was stretched to baseline tension of 80-100g for 30 seconds. The rack and pinion was rapidly turned to achieve a tension of 750g and held for 30 seconds. Tendon stretch was used to simulate the mechanical deformation that occurs with contracting skeletal muscle. Blood pressure and heart rate were given 5 minutes to stabilize after the stretch maneuver. The snare was tightened to occlude blood flow out of the hindlimb and 30 seconds were given to allow stabilization of blood pressure. Random order of injection into the superficial epigastric artery of 0.2 mL of lactic acid (24 mM; Sigma Aldrich, St. Louis, MO) and 0.2 mL of α,β-Methylene ATP (20μg/kg; Tocris Bioscience, Bristol, UK) were given; injections were separated by 10 minutes. Post-injection the snare was released and the catheter was flushed with saline. In a separate group of rats (n=3), the snare was tightened and 0.1 mL of GsMTx-4 (10μg/100μl; Alomone Labs, Jerusalem, Israel), a selective mechano-gated Piezo channel inhibitor, were injected into the arterial supply of the hindlimb. The snare was held in place for 10 minutes to trap the injectate in the hindlimb. After the release of the snare, we waited 20 minutes before reproducing the protocol; after the stretch maneuver, the order of injection was the same as the initial injection. At the end of the experiment 0.2 mL of Evans blue dye (Sigma Aldrich, St. Louis, MO) was injected into the arterial supply of the leg with the snare tightened to ensure the injectate was administered to the targeted musculature. This was confirmed when the triceps surae turned blue.

### STATISTICAL ANALYSES

Offline analysis of Spike2 data consisted of analyzing the script of every rat as follows: peak responses of mean arterial pressure and heart rate were taken during 30

seconds of baseline tension and during 30 seconds following the stretch maneuver or injection of metabolites. Ultimately, the change in mean arterial pressure and heart rate were calculated by taking the difference between the peak response during stretch/injection and the peak response during baseline. Tension-time indexes (TTI) for stretch were calculated by taking difference of the area under the curve of the developed tensions during 30 seconds of baseline and 30 seconds of the stretch maneuver.

Data are presented as means ± S.E.M. Comparisons of body weight, glucose, and HbA1c measurements between CTL and STZ rats were analyzed using unpaired t-test. The pressor responses, cardioaccelerator responses, and tensions developed from tendon stretch were compared using unpaired t-test. Pressor responses and cardioaccelerator responses from injection of metabolites and stretch, as well as tensions developed from tendon stretch before and after GsMTx-4 injection in the same animals were compared using paired t-test. $P < 0.05$ established statistical significance.

# Chapter 3: Results

## DESCRIPTIVE CHARACTERISTICS

Sprague-Dawley rats of either sex were experimented on (n=15). They were randomly put into the control group (CTL; n=6) or the streptozotocin-induced diabetic group (STZ; n=9). Table 1 shows the descriptive characteristics of the rats. Before injection of STZ or the vehicle, CTL rats (336.2 ± 22.12 g) were significantly heavier than STZ rats (239.6 ± 6.69), $p<0.05$. Similar comparisons were seen on the day of experiment in which CTL rats (351.5 ± 21.01) were significantly heavier than STZ rats (251.2 ± 11.47), $p<0.05$. It is important to note that although these differences occurred, the change in weight in CTL rats (15.33 ± 15.66) was not significantly different from the STZ rats (11.67 ± 8.05), $p>0.05$. Therefore, the change in weight was the same across both groups.

Blood glucose levels and HbA1c levels were used to determine a diabetic state. Diabetes was determined by blood glucose levels greater than 300mg/dL and HbA1c levels greater than 6.5%. Prior to injection, blood glucose and HbA1c levels were the statistically the same (Table 1). On the day of experiment, CTL rats (176.3 ± 10.94) had significantly lower blood glucose in comparison to the STZ rats (468.1 ± 23.18), $p<0.05$. Additionally, HbA1c levels in CTL rats (4.23 ± 0.12) were significantly lower than STZ rats (6.09 ± 0.20), $p<0.05$.

## RESPONSES TO TENDON STRETCH

Pressor and cardioaccelerator responses elicited by tendon stretch can be seen in Figure 1. STZ rats (42.11 ± 8.34) showed a greater change in mean arterial pressure in comparison to CTL rats (18.67 ± 4.04), $p<0.05$. Unlike pressor responses, change in

cardioaccelerator responses in CTL rats (9.67 ± 2.69) and STZ rats (13.67 ± 3.69) were not different, $p>0.05$. Figure 1 shows tensions developed by tendon stretched, which were similar in both CTL rats and STZ rats, $p>0.05$. Figure 2 shows raw data tracings of blood pressure, heart rate, mean arterial pressure, and tension during 30 seconds of baseline tension and 30 seconds of tendon stretch in a CTL rat and STZ rat, respectively. Baseline pressor, cardioaccelerator, and tension values were not significantly different in CTL and STZ rats.

### INHIBITION OF PIEZO 1 & 2 CHANNELS

In a subset group of STZ rats (n=3), tendon stretch was done before and after injecting GsMTx-4 into the arterial supply of the hindlimb. Pressor responses and cardioaccelerator responses as well as the tension developed before and after injecting GsMTx-4 can be seen in Figure 3. Pressor responses elicited by tendon stretch before injection (65.33 ± 4.98) were significantly greater than after injection (46 ± 4.16) of GsMTx-4, $p<0.05$. Cardioaccelerator responses to tendon stretch before injection of GsMTx-4 (16.67 ± 4.18) were not different after injection (13.33 ± 6.77), $p>0.05$. The tensions developed from tendon stretch were similar before and after injection of GsMTx-4, $p>0.05$. Figure 4 shows no differences in the pressor and cardioaccelerator responses elicited by lactic acid injection before and after injection of GsMTx-4, $p>0.05$. Similarly, Figure 5 shows no differences in pressor and cardioaccelerator responses elicited by α,β-Methylene ATP injection before and after injection of GsMTx-4, $p>0.05$. Figure 6 shows raw data tracings of blood pressure, heart rate, mean arterial pressure, and tension during 30 seconds of baseline tension and 30 seconds of tendon stretch before injection of GsMTx-

4 and after injection. Baseline pressor, cardioaccelerator, and tension values were not significantly different before and after injection of GsMTx-4.

**Table 1:** Descriptive characteristics of rats.

| | CTL n = 6 | | | STZ n = 9 | | |
|---|---|---|---|---|---|---|
| | **Before Injection** | **Day of Experiment** | **Δ** | **Before Injection** | **Day of Experiment** | **Δ** |
| **Body weight (g)** | 336.2 ± 22.12 | 351.5 ± 21.01 | 15.33 ± 15.66 | 239.6 ± 6.69* | 251.2 ± 11.47* | 11.67 ± 8.05 |
| **Glucose (mg/dL)** | 131.5 ± 16.65 | 176.3 ± 10.94 | 44.83 ± 22.54 | 107.2 ± 9.88 | 468.1 ± 23.18* | 360.9 ± 30.13* |
| **HbA1c (%)** | 4.08 ± 0.08 | 4.23 ± 0.12 | | 4.23 ± 0.13 | 6.09 ± 0.20* | |

*Significantly different from CTL ($p < 0.05$).

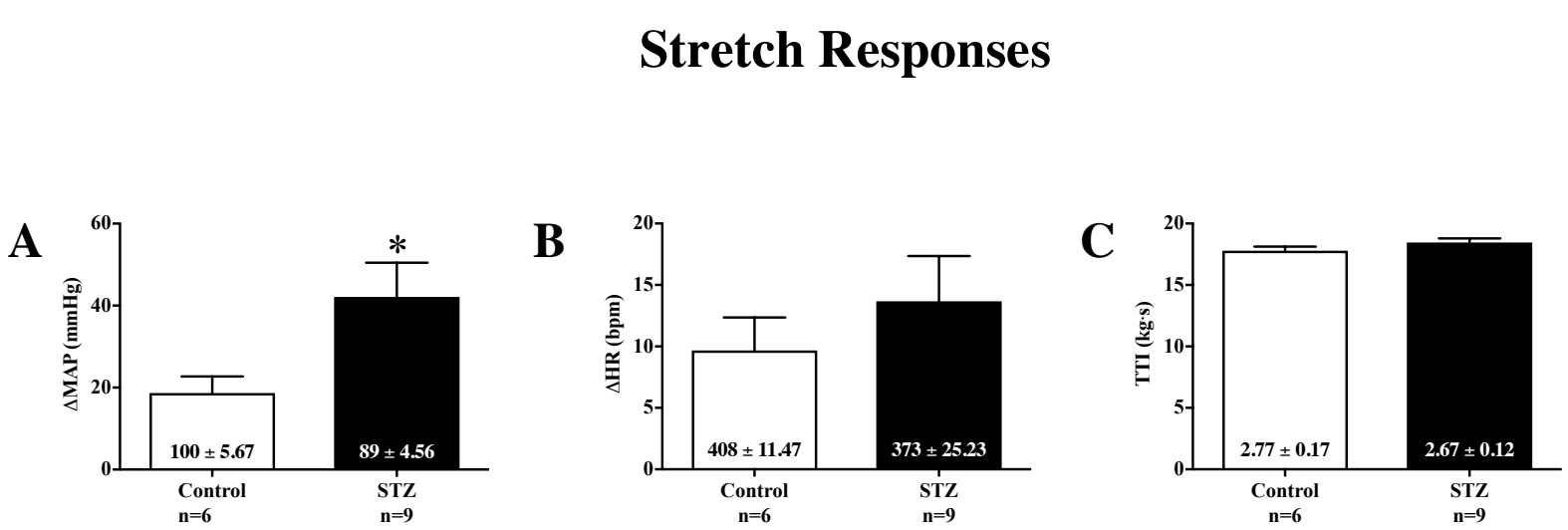


**Figure 1:** Pressor (**A**) and cardioaccelerator (**B**) responses evoked by tendon stretch in control and STZ rats. Similar tensions were developed by tendon stretch in control and STZ rats (**C**). (*) Significantly greater pressor response compared to control ($p < 0.05$). Numbers within mean bars are the corresponding baseline values.

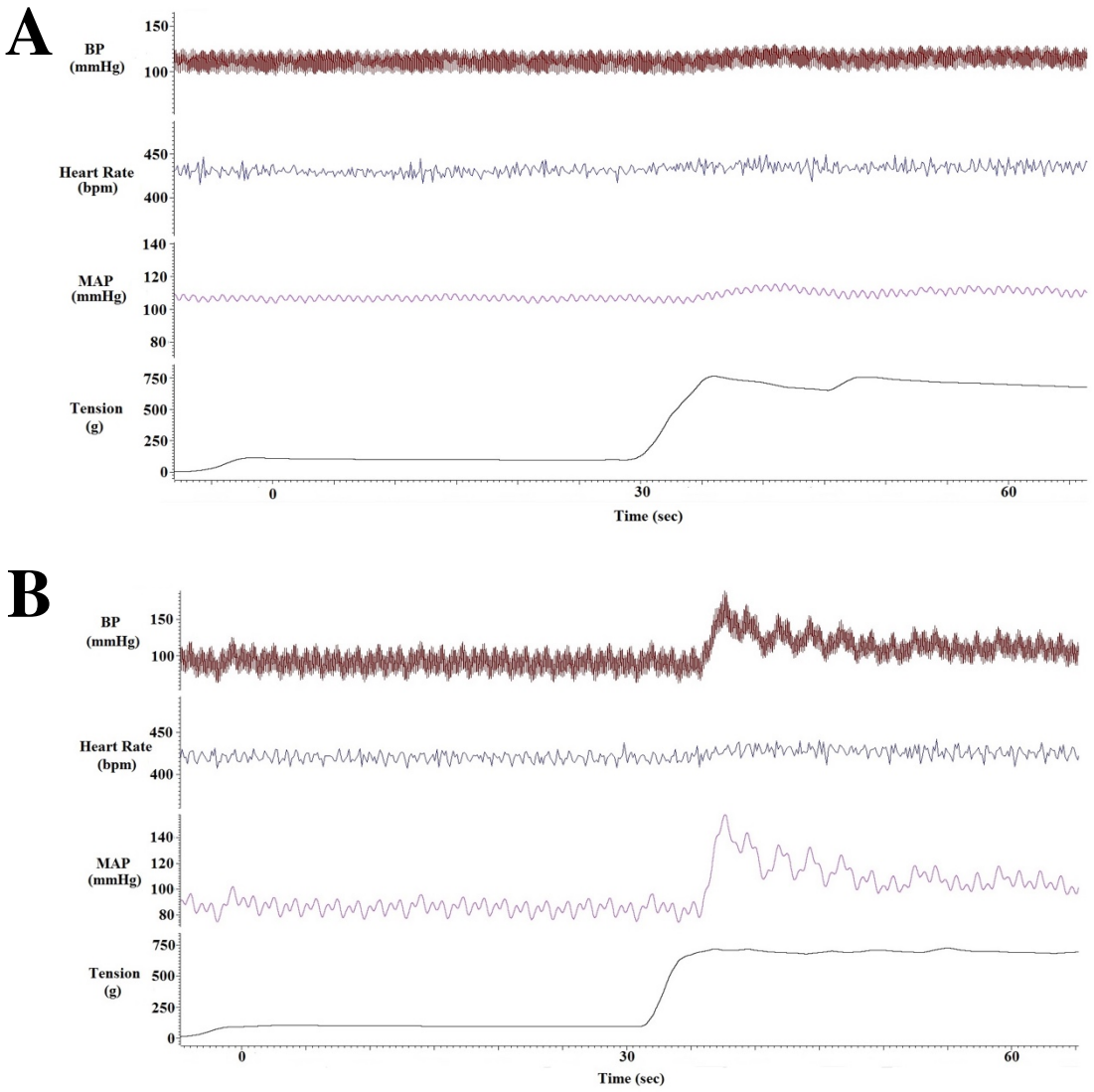


**Figure 2:** Raw data tracing of blood pressure, heart rate, mean arterial pressure, and tension (top to bottom) during 30 seconds of baseline tension and 30 seconds of a tendon stretch in a control (**A**) and STZ (**B**) rat.

# Inhibition of Piezo 1 & 2 channels

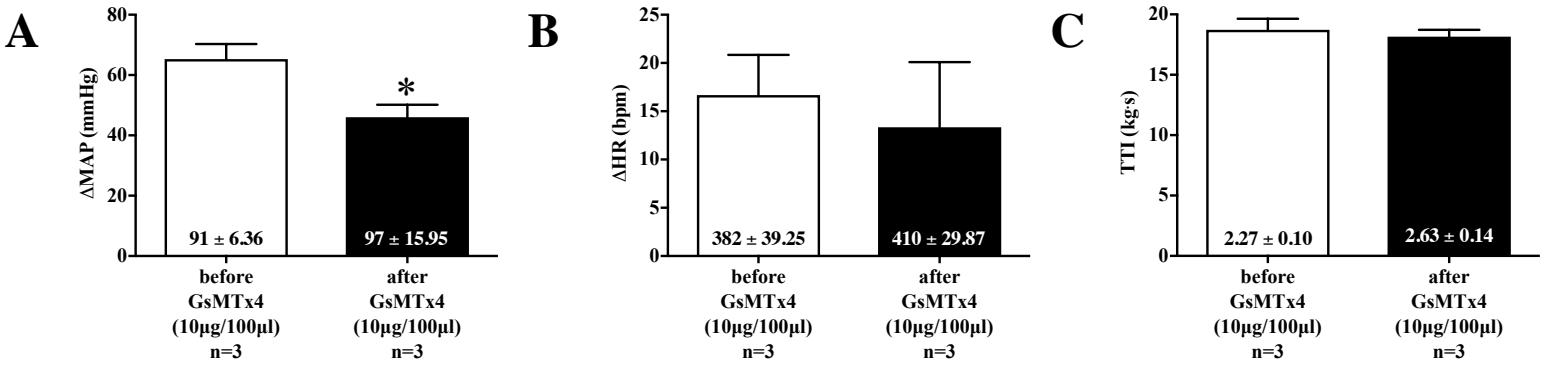


**Figure 3:** Pressor (**A**) and cardioaccelerator (**B**) responses evoked by tendon stretch before and after injecting GsMTx-4 in STZ rats. Similar tensions were developed by tendon stretch in STZ rats before and after injecting GsMTx-4 (**C**). (*) Significantly smaller pressor response compared to control ($p < 0.05$). Numbers within mean bars are the corresponding baseline values.

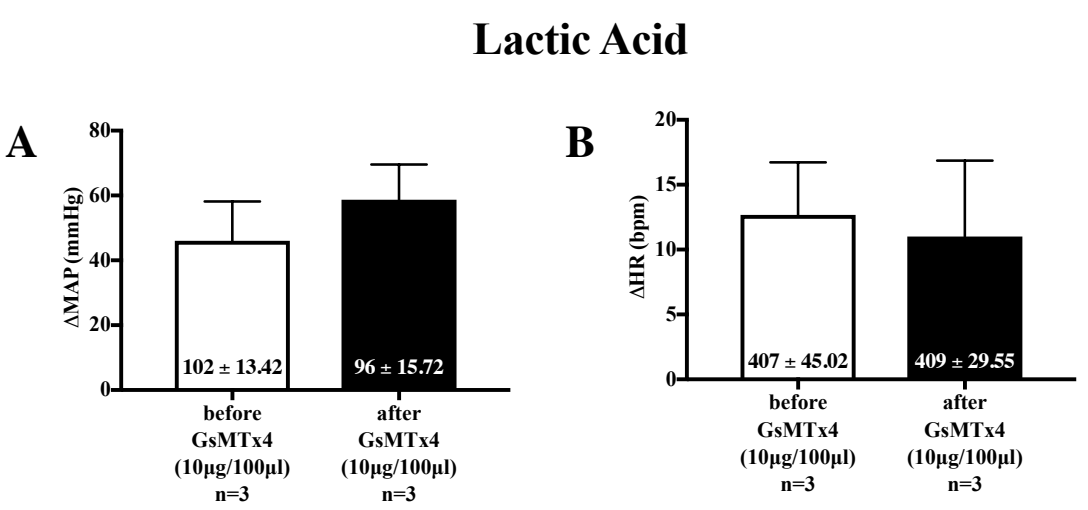


**Figure 4:** Pressor (**A**) and cardioaccelerator (**B**) responses evoked by injecting lactic acid into the arterial supply of the hindlimb before and after injecting GsMTx-4 in STZ rats ($p > 0.05$). Numbers within mean bars are the corresponding baseline values.

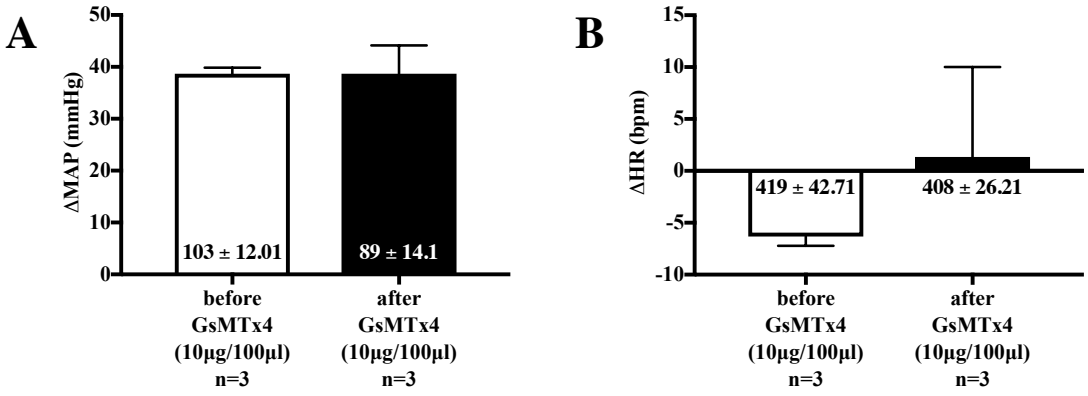


**Figure 5:** Pressor (**A**) and cardioaccelerator (**B**) responses evoked by injecting α,β-Methylene ATP into the arterial supply of the hindlimb before and after injecting GsMTx-4 in STZ rats ($p > 0.05$). Numbers within mean bars are the corresponding baseline values.

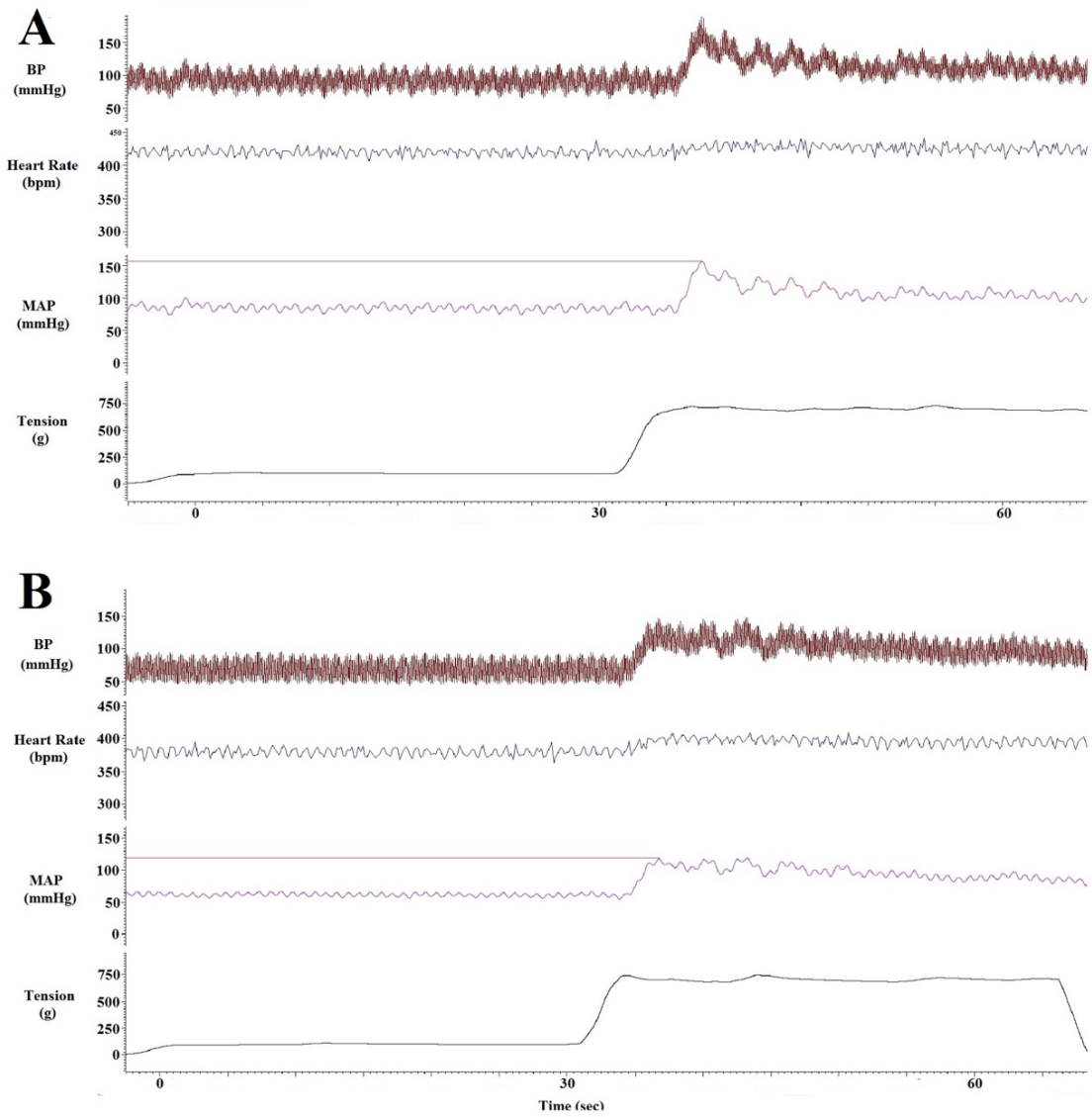


**Figure 6:** Raw data tracing of blood pressure, heart rate, mean arterial pressure, and tension (top to bottom) during 30 seconds of baseline tension and 30 seconds of a tendon stretch in an STZ rat before injecting GsMTx-4 (**A**) and after injecting GsMTx-4 (**B**). Red line drawn on MAP is to show the attenuation seen in the pressor response.

# Chapter 4: Discussion

The primary finding of this study was that the mechanoreflex was exaggerated in the early stages of streptozotocin-induced T1DM in rats. Furthermore, inhibition of mechanically-sensitive Piezo 1 and 2 channels attenuated the exaggerated responses elicited by tendon stretch. Exercise plays an important role in type 1 diabetic patients because it has been shown to reduce their incidence of cardiovascular disease (Tikkanen-Dolenc et al., 2017). If the responses to exercise are exaggerated, these may predispose this patient population to a cerebral and/or cardiovascular event. Since cardiovascular disease commonly causes early morbidity and mortality in this patient population (Morrish et al., 2001), their ability to exercise is crucial.

These findings are in line with what has been seen in other diseases such as heart failure, hypertension, and peripheral artery disease. In a dilated cardiomyopathy rat model, used to simulate heart failure, there was an augmented pressor response to stretch and contraction; simultaneously, as the severity of left ventricular dysfunction increased, the augmented pressor response increased (Smith et al., 2003). As stretch and contraction without occlusion solely stimulate mechanoreceptors, this suggests that Group III afferents are over sensitized and the mechanoreceptors on these afferents have an enhanced responsiveness and/or expression. Likewise, in hypertensive rats similar findings have been seen. Stimulation of both the mechanoreflex and metaboreflex showed augmented pressor responses in spontaneously hypertensive rats in comparison to normotensive rats. Interestingly, augmented pressor responses induced by stretch were positively correlated with increasing tensions (Leal et al., 2008). More importantly, the augmented pressor responses present at low intensities were four times greater than those seen in normotensive rats. Moreover, in a rat model of peripheral artery disease, which is simulated by chronic

ligation of the femoral artery, pressor responses to intermittent contraction were greater than those with freely perfused femoral arteries. Furthermore, the use of selective mechano-gated channel inhibitor GsMTx-4 before and after stimulation of the mechanoreflex showed an attenuation of the exaggerated pressor response seen in the ligated rats (Copp et al., 2016b). Similar to those findings, the current study showed that the mechanoreflex contributes to the exaggerated responses in the exercise pressor reflex in T1DM.

In the present study, stimulation of the mechanoreflex in diabetic rats showed augmented pressor responses in comparison to healthy controls. I next wanted to determine the role of Piezo channels in evoking the mechanoreflex in T1DM rats. We found that inhibiting the Piezo channels with GsMTx-4 significantly attenuated the pressor response evoked by tendon stretch. This suggests that Piezo channels play a significant role in the mechanoreflex in rats. Furthermore, the pressor responses to arterial injections of α,β-Methylene ATP or Lactic Acid were not attenuated by GsMTx-4. This suggests that the GsMTx-4 was specifically inhibiting the mechanoreflex and not the metaboreflex. Dorsal root ganglion (DRG) samples were harvested but not processed. Future studies are needed to determine the quantity of Piezo 1 and 2 proteins in dorsal root ganglia whose peripheral endings terminate in the hindlimb muscles.

One limitation seen in this study was the sample size obtained, specifically in the second hypothesis. Although the differences were statistically significant, the sample size was small and further collection of data needs to be done to establish statistical power. A second limitation was the difference in the initial bodyweight between the CTL and STZ rats. CTL rats were significantly larger than STZ rats which might suggest that they differed in age. We do not think that age played a large role in this study since rats typically grow very fast and can significantly differ in weight in one week's time. It is important to

note that their change in bodyweight before injection and on the day of experiment were not different, and all rats were used for the experiment one week after injection.

In conclusion, this study showed that T1DM exaggerated the mechanoreflex in rats. Furthermore, this exaggeration was attenuated when mechano-gated Piezo 1 and 2 channels were inhibited. Future studies should attempt to quantify Piezo 1 and 2 channels through methods such as Western blot analyses. Additionally, it would be interesting to correlate the exaggerated pressor response to tendon stretch with the pain responses to Von Frey filaments in T1DM rats.

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