GsMTx4 reduces the pressor response during dynamic hindlimb skeletal muscle stretch in decerebrate rats

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Abstract

Mechanical signals within contracting skeletal muscles contribute to the generation of the exercise pressor reflex; an important autonomic and cardiovascular control mechanism. In decerebrate rats, GsMTx4, a mechanically–activated channel inhibitor that is partially selective for piezo channels, was found recently to reduce the pressor response during static hindlimb muscle stretch; a maneuver used to investigate the mechanical component of the exercise pressor reflex (i.e., the mechanoreflex). However, the effect was found only during the very initial phase of the stretch when muscle length was changing which may have reflected the inhibition of rapidly-deactivating piezo 2 channels and the fact that different mechanically-activated channels with slower deactivation kinetics evoked the pressor response during the static phase of the maneuver. We tested the hypothesis that in decerebrate, unanesthetized rats, GsMTx4 would reduce the pressor response throughout the duration of a 30 second, 1 Hz dynamic hindlimb muscle stretch protocol. We found that the injection of 10 μg of GsMTx4 into the arterial supply of a hindlimb reduced the peak pressor response (control: 15±4, GsMTx4: 5±2 mmHg, $p<0.05$, n=8) and the pressor response at multiple time points throughout the duration of the stretch. GsMTx4, however, had no effect on the pressor response to the hindlimb arterial injection of lactic acid. Moreover, the injection of GsMTx4 into the jugular vein (a systemic control, n=5) or the injection of saline into the hindlimb arterial supply (a vehicle control, n=4) had no effect on the pressor response during dynamic stretch. We conclude that GsMTx4 reduced the pressor response throughout the duration of a 1 Hz dynamic stretch protocol which may have reflected the inhibition of piezo 2 channels throughout the dynamic stretch maneuver.

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Acknowledgements

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Preface

Mechanotransduction is the process by which cells translate mechanical signals into electrochemical signals. This process is conserved across cell types and species, from plants to animals, and is responsible for many facets of biological function. In humans and animals, mechanotransduction is foundational for the senses of touch, proprioception, balance, and hearing. Beyond conscious sensation however, mechanotransduction also plays a vital role in intrinsic sensation, including sensation of vascular shear stress and bladder distension.

There are many processes that mediate mechanotransduction including conformational changes in epithelial cell membranes and disruption of integrins or cadherins; however, one of the primary methods of mechanotransduction is the opening of mechanically-activated channels. When mechanical deformation of a cell occurs, mechanically-activated ion channels change configuration to allow ion flux across the cell membrane, thus changing the membrane potential of the cell. This is the method by which mechanical signals from within contracting skeletal muscles are converted into neural impulses that travel to the brainstem to promote the autonomic and cardiovascular adjustments to exercise (i.e., the mechanoreflex).

In 2010, a new class of mechanically-activated channel was discovered and named piezo (from the Greek "πίεση" (piesi), meaning pressure) (11). In the years since their discovery, piezo channels have been implicated as foundational in many physiological mechanotransduction processes. Piezo 2 channels specifically, which are highly expressed in sensory neurons, appear to be essential for the transduction of mechanical signals from contracting skeletal muscles into the afferent neural signals of the mechanoreflex. It is this prospect that serves as the foundation of this thesis.

Chapter 1 - Introduction

The exercise pressor reflex is activated when mechanical and metabolic signals arising from within contracting skeletal muscles stimulate the sensory endings of group III and group IV muscle afferents (36). These feedback signals contribute to increases in sympathetic nervous system activity, heart rate, and blood pressure which facilitates increased perfusion of contracting skeletal muscle. This reflex was classically considered an ischemically-activated reflex that increased perfusion pressure when oxygen supply could not meet contracting skeletal muscle oxygen demand (43). Based on this conventionally-held notion, the molecular basis of the metabolically-sensitive component of the reflex (i.e., the metaboreflex) has long been the focus of significant research efforts. More recently, there has been an increased focus on the mechanically-sensitive component of the reflex (i.e., the mechanoreflex) (2, 9, 12, 15, 17, 21, 25). This has been driven, in large part, by findings that mechanoreflex alterations contribute to the exaggerated pressor response during exercise present in multiple forms of cardiovascular disease (10, 26, 28-30, 33, 34, 38, 45, 46).

Coste et al. (11) recently identified and described a ubiquitously expressed class of mechanically-activated channel they named “piezo” along with the specific subclasses piezo 1 and piezo 2. That report, in conjunction with the finding that the tarantula venom peptide GsMTx4 inhibited piezo channels with at least some degree of selectivity over other classes of mechanically-activated channels (4), prompted recent investigation of the mechanistic underpinnings of the mechanoreflex activation. Specifically, Copp et al. (9) found that in decerebrate, unanesthetized rats GsMTx4 reduced the pressor response during static hindlimb skeletal muscle stretch (a model of selective activation of the mechanoreflex during static exercise) and dynamic hindlimb muscle contractions. Moreover, piezo1 and piezo2 channel

expression were confirmed in L_4 and L_5 rat dorsal root ganglia (DRG) tissue (9). Together, those findings supported the possibility that piezo channels contributed to the activation of the mechanoreflex and the exercise pressor reflex in the rat. A nuanced finding of that investigation was that GsMTx4 reduced the pressor response during the first ~five seconds of the 30 second static stretch protocol when muscle length was changing rapidly whereas there was no effect on the pressor response during the later phases when muscle length was relatively constant. In contrast, GsMTx4 reduced the pressor response throughout the duration of the 30 second dynamic contraction protocol (9). In discussion of those findings, the speculation was raised that different classes of mechanically-activated channels mediated the pressor response during the different phases of static stretch and that GsMTx4 acted primarily on the channels that mediated the pressor response during the initial phase when muscle length was changing. This is at least plausible given the partial selectivity of GsMTx4 for piezo channels (4) and the rapid deactivation kinetics of piezo channels, especially piezo 2 channels (11).

The purpose of this investigation was to further explore the above speculation by testing the hypothesis that GsMTx4 would reduce the pressor response throughout the duration of a 1 Hz dynamic hindlimb skeletal muscle stretch protocol in decerebrate rats. Our laboratory recently used such a protocol, a maneuver which produces repeated changes in muscle length in the absence of contraction-induced metabolite production, as an experimental tool to investigate the activation of the mechanoreflex during dynamic muscle contractions (26).

Chapter 2 - Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on young adult male Sprague-Dawley rats (n=17, average body weight = 385±10 g). The rats were housed two per cage in temperature (maintained at ~22 degrees C) and light (12/12 hour light/dark cycle running from 7am to 7pm) controlled accredited facilities with standard rat chow and water provided *ad libitum*. At the end of each experiment, the decerebrated rats (see below) were euthanized with an intravenous injection of saturated (>3 mg/kg) potassium chloride.

Surgical Procedures. All rats were anesthetized initially with 5% isoflurane (balance O_2). Depth of anesthesia was confirmed by an absent toe-pinch reflex. A tracheostomy was performed, and rats' lungs were mechanically ventilated (Harvard Apparatus) with gaseous anesthetic (2% isoflurane balance O_2). The right jugular vein and both common carotid arteries were cannulated with PE-50 catheters for administration of drugs, measurement of arterial blood pressure (Physiological Pressure Transducer, AD Instruments), and sampling of arterial blood gases. In 12 rats, a reversible snare (2-0 suture) was placed around the left common iliac artery and vein just distal to the descending aorta/inferior vena cava. The left superficial epigastric artery was cannulated with a PE-8 catheter for administration of drugs. In all rats, the left calcaneus bone was severed and the triceps surae (gastrocnemius, soleus, and plantaris) muscles were exposed. The triceps surae muscles were then connected by string to a force transducer (Grass FT03) attached to a rack and pinion.

Upon completion of the initial surgical procedures, all rats were secured in a Kopf stereotaxic frame. After administering dexamethasone (0.2 mg i.v.) to minimize swelling of the brainstem, a decerebration was performed in which all brain tissue rostral to the superior colliculi was removed. Following decerebration, anesthesia was terminated, and the rats' lungs were ventilated with room air. All rats were allowed a minimum of 60 minutes to recover from anesthesia before initiation of any experimental protocol. Body core temperature was measured via rectal probe and maintained at ~37-38 degrees Celsius by an automated heating system (Harvard Apparatus) and heat lamp. Arterial pH and blood gases were analyzed periodically throughout the experiment from small blood samples (~75 uL) and maintained within physiological ranges (pH: 7.35-7.45; pCO_2: ~38-40 mmHg; PO_2: ~100 mmHg) by administration of sodium bicarbonate and/or adjusting ventilation.

Experimental protocols: In eight rats, we compared the increase in blood pressure during a 1 Hz dynamic stretch protocol of the triceps surae muscles and following injection of 24 mM lactic acid before and after intra-arterial injection of GsMTx4 into the hindlimb circulation. Prior to the experimental protocol, all rats were paralyzed with injection of pancuronium bromide (~1 mg/kg i.v.). Baseline muscle tension was set at ~100 g of tension and blood pressure and heart rate were collected for ~30 seconds. The triceps surae muscles were then stretched for 30 seconds by manually turning the rack and pinion at a 1 Hz frequency by an experienced investigator who followed the cadence of a metronome. Approximately five minutes following the completion of the stretch protocol and ensuring that blood pressure had returned to its pre-stretch baseline value, we injected lactic acid (0.2 mL of 24 mM concentration in saline) into the arterial supply of the hindlimb via the superficial epigastric artery catheter. After another approximately five minute waiting period and after ensuring blood pressure once again returned

to baseline, we tightened the iliac snare to occlude flow to and from the hindlimb and injected GsMTx4 (10 ug dissolved in 0.2 mL of saline) into the arterial supply of the hindlimb via the superficial epigastric artery catheter. The drug remained snared in the hindlimb circulation for 10 minutes, at which time the iliac snare was released and the leg was allowed to reperfuse for 20 minutes. After reperfusion, the dynamic stretch and lactic acid injection protocols were repeated exactly as described above with care being taken to develop similar tension during the post-GsMTx4 stretch to that produced during the control stretch. Evan's blue dye was then injected into the arterial supply of the hindlimb in the same manner that GsMTx4 was injected to ensure that the peptide had access to the triceps surae muscle circulation in all experiments. The triceps surae muscles were observed to stain blue in all experiments.

In an additional group of five rats, we compared the pressor response during dynamic stretch before and after i.v. administration of GsMTx4. We performed the same stretch protocol as described above, except that 10 ug of GsMTx4 was injected into the jugular vein. In another group of four rats, we compared the pressor response during dynamic stretch before and after intra-arterial administration of saline (0.2 ml) with snare, the vehicle for GsMTx4, via the superficial epigastric artery catheter.

Data and statistical analysis: Arterial blood pressure and muscle tension were measured (PowerLab and LabChart data acquisition; AD Instruments) and mean arterial pressure (MAP) and heart rate (HR) were calculated in real time and recorded for offline analysis. Baseline MAP and HR were determined for the 30 second baseline periods that preceded each stretch maneuver (stretch or lactic acid injection). The peak increase in MAP (peak Δ MAP) and HR (peak Δ HR) during dynamic stretch or lactic acid injection was calculated as the difference between the peak values wherever they occurred during the maneuvers and the corresponding baseline value. The

time course of the increase in MAP was plotted as the Δ MAP from baseline over the course of the 30 second stretch protocols. The change in the tension time integral (Δ TTI) during stretch above baseline was calculated by integrating the area under the tension signal and subtracting the integrated area during the baseline period.

Data are expressed as mean±SE. Data were compared with paired Student's t-tests or repeated measures ANOVAs with Holm-Sidak post-hoc tests as appropriate. Statistical significance was defined as $p<0.05$.

Chapter 3 - Results

Thirty seconds of 1 Hz dynamic hindlimb muscle stretch markedly increased MAP and HR. The peak increase in MAP occurred, on average, nine seconds following the onset of stretch and MAP remained elevated above baseline for the duration of the maneuver. In eight rats, the injection of GsMTx4 into the arterial supply of the hindlimb significantly reduced the pressor response during dynamic stretch; an effect that was evident at multiple time points throughout the duration of the maneuver (Figure 1A). Moreover, compared to control, GsMTx4 significantly reduced the peak pressor response during dynamic stretch (Figure 1B) whereas the effect on peak HR did not reach statistical significance (control: 11±2, GsMTx4: 6±2 bpm, p=0.07). There were no differences in the Δ TTI between control and GsMTx4 conditions (Figure 1C). In these same rats in which we performed dynamic stretch protocols, we also injected lactic acid into the arterial supply of the hindlimb before and after the injection of GsMTx4. We found that GsMTx4 had no effect on the peak pressor response or the time course of the pressor response that resulted from the injection of lactic acid (Figure 2) which is consistent with the notion that GsMTx4 did not have off-target effects on skeletal muscle sensory neurons such as the inhibition of voltage-gated sodium (NaV) channels (42).

In an additional group of five rats, we compared the pressor response during dynamic stretch before and after the injection of 10 μg of GsMTx4 into the jugular vein to determine if the effects observed when injected into the hindlimb arterial supply could be attributed to systemic effects elsewhere in the mechanoreflex arc such as the spinal cord or the medulla of the brainstem, for example. The injection of GsMTx4 into the jugular vein had no effect on the time course of the pressor response, the peak pressor response (Figure 3A and 3B), or the peak HR response (control: 12±6, GsMTx4: 10±3 bpm, p=0.79) during dynamic stretch. Likewise, in four

additional rats, the injection of saline (the vehicle for GsMTx4) into the arterial supply of the hindlimb had no effect on the time course of the pressor response, the peak pressor response (Figure 4A and 4B), or the peak HR response (control: 13±5, GsMTx4: 9±3 bpm, p=0.17) during dynamic stretch. There were no differences in the Δ TTI or the average peak Δ tension of the stretches between conditions for either the i.v. injection (Figure 3C) or saline (Figure 4C) control experiments.

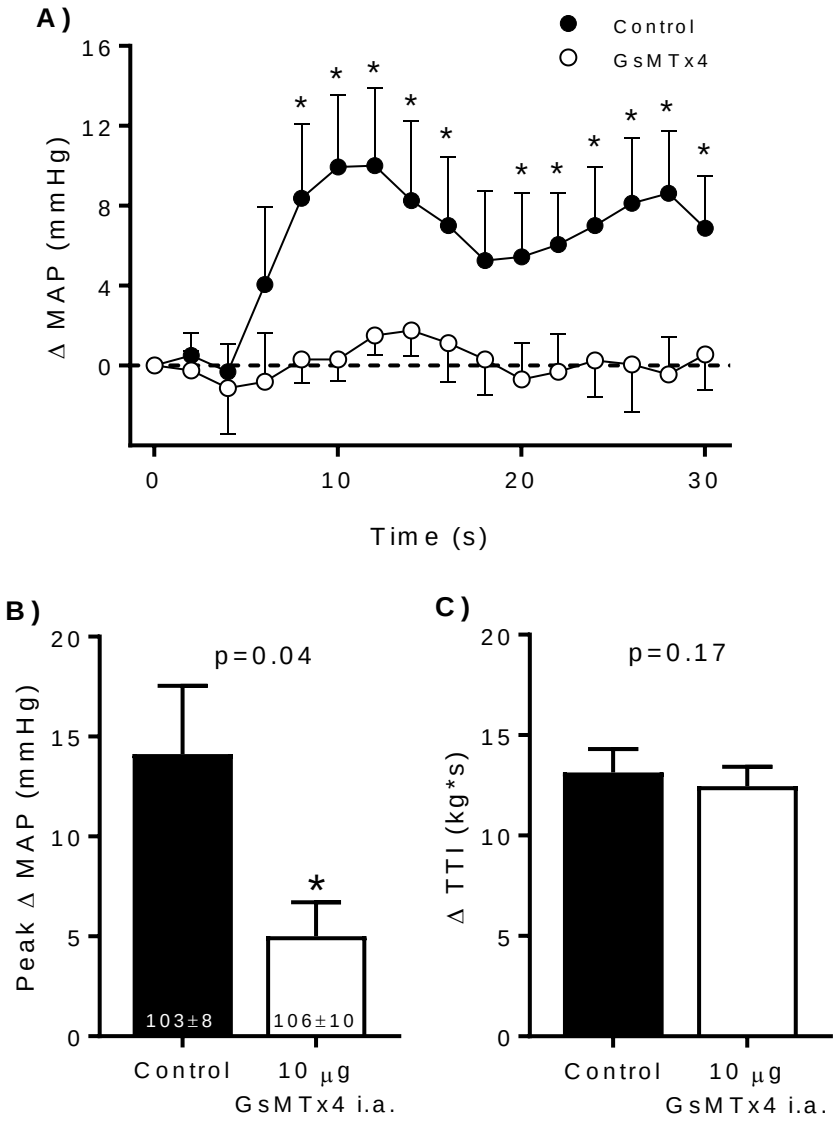

Figure 1. The effect of hindlimb intra-arterial (i.a.) GsMTx4 injection on blood pressure during dynamic stretch

Injection of 10 μg of GsMTx4 into the arterial supply of the hindlimb (n=8) significantly reduced the pressor response at multiple time points throughout the duration of dynamic hindlimb muscle stretch. Baseline blood pressure values, indicated at the base of the bars in panel B, were not significantly different between conditions. TTI=tension time integral, *$p<0.05$

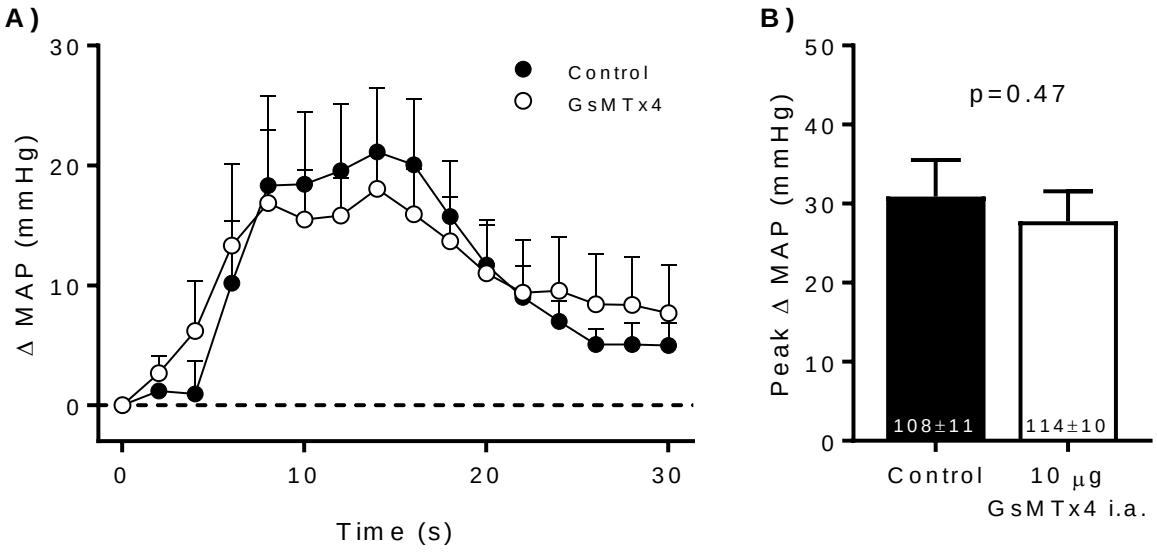

Figure 2. The effect of hindlimb intra-arterial (i.a.) GsMTx4 injection on blood pressure following lactic acid injection

Injection of 10 μg of GsMTx4 into the arterial supply of the hindlimb (n=8) had no effect on the pressor response to lactic acid injection (0.2 mL, 24 mM), indicating that GsMTx4 did not exert off-target effects. Baseline blood pressures, indicated at the base of the bars in panel B, were not significantly different between conditions.

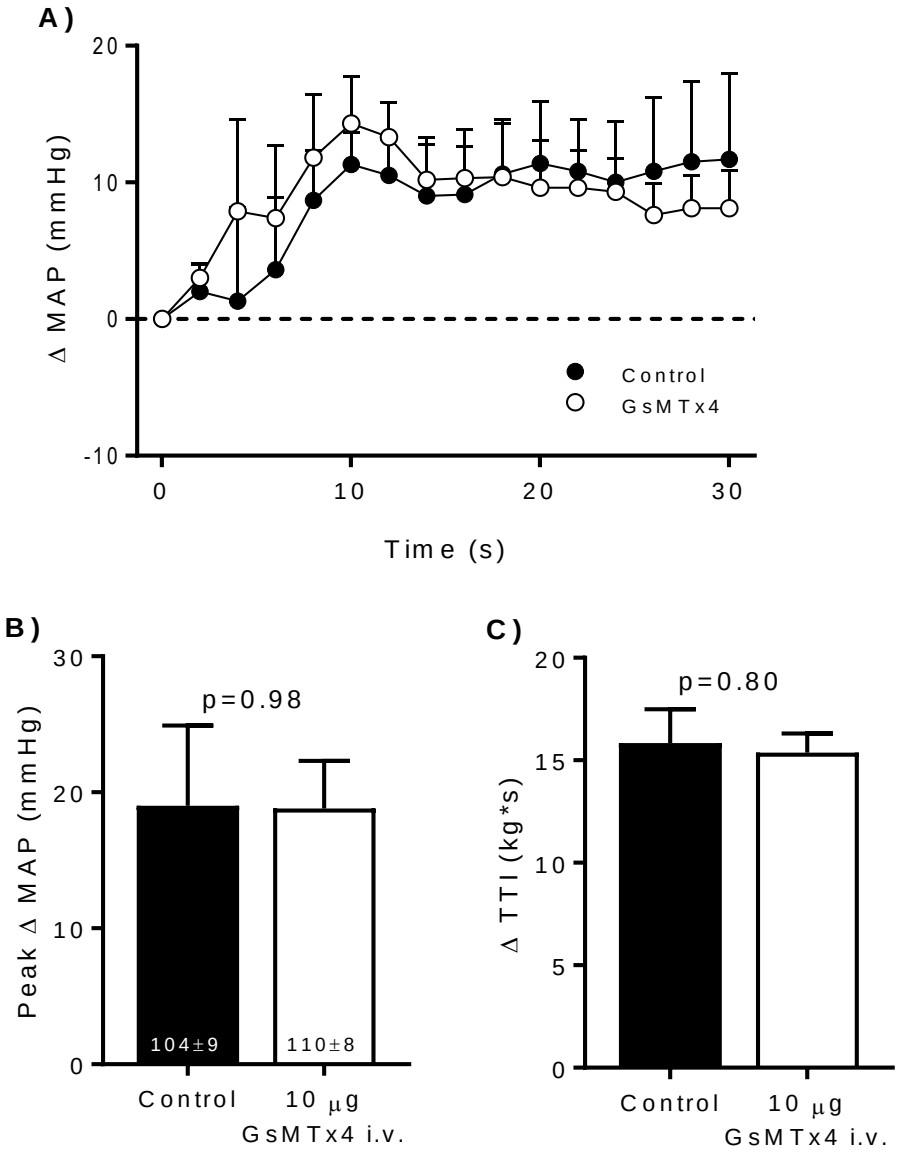

Figure 3. The effect of intravenous (i.v.) GsMTx4 injection on blood pressure during dynamic stretch

Injection of 10 μg of GsMTx4 into the jugular vein (n=5) had no effect on the pressor response during dynamic hindlimb muscle stretch, indicating that GsMTx4 exerted its effects locally in the experimental group. Baseline blood pressures, indicated at the base of the bars in panel B, were not significantly different between conditions. TTI=tension time integral

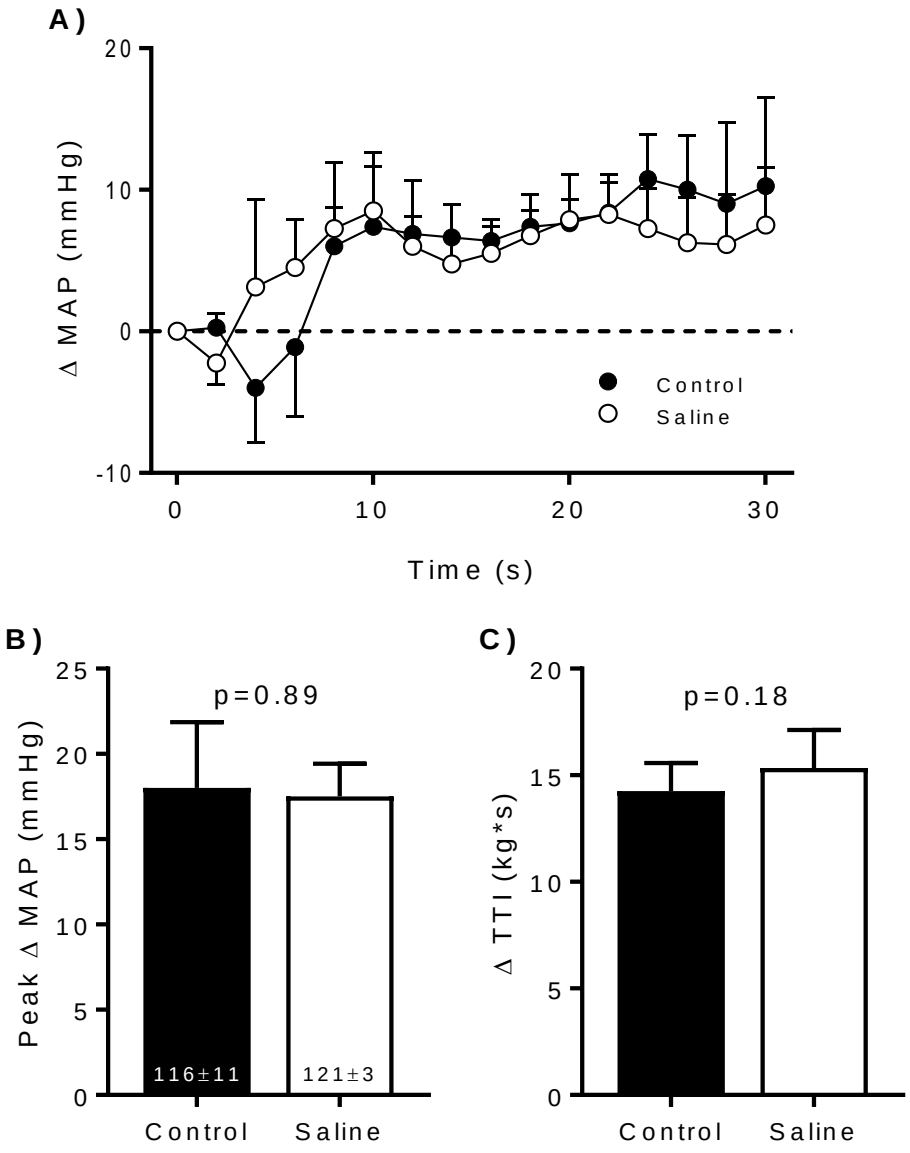

Figure 4. The effect of hindlimb intra-arterial (i.a.) saline injection on blood pressure during dynamic stretch

Injection of saline into the arterial supply of the hindlimb (n=4) had no effect on the pressor response during dynamic hindlimb muscle stretch, indicating no vehicle effects. Baseline blood pressures, indicated at the base of the bars in panel B, were not significantly different between conditions. TTI=tension time integral

Chapter 4 - Discussion

We tested the effects of GsMTx4, a mechano-gated channel inhibitor that is relatively selective for piezo channels, on the pressor response during 1 Hz dynamic hindlimb muscle stretch. In confirmation of our hypothesis, we found that the hindlimb arterial injection of GsMTx4 reduced the pressor response throughout the duration of a dynamic stretch protocol in a similar fashion to the reductions seen in dynamic contraction (9). However, GsMTx4 had no effect on the pressor response to intra-arterial injection of 24 mM lactic acid, which indicates that GsMTx4 did not exert non-specific effects such as blockade of $NaV_{1.7}$ channels and therefore inhibit the transmission of the mechanoreflex signals along the axons of the mechanically-sensitive muscle afferents. Furthermore, i.v. injection of GsMTx4 did not affect the pressor response during dynamic stretch, indicating that the toxin did not exert systemic effects elsewhere in the mechanoreflex arc. Finally, intra-arterial injection of saline as a vehicle control had no effect on the pressor response during dynamic stretch. Collectively, therefore, our experimental controls indicate that the reduction of the pressor response during dynamic stretch found following the injection of GsMTx4 into the arterial supply of the hindlimb was due to the peptide's effect on the sensory endings of the mechanically-sensitive muscle afferents and not local or systemic off-target effects or an effect of time such as the deterioration of the experimental preparations.

The mechanoreflex has long been understudied. Initially, this was due to the belief that the mechanoreflex did not contribute importantly to exercise pressor reflex activation. More recently, the mechanoreflex has been found to play a very important role in exercise pressor reflex activation (16), leading to an increased realization of the need to study it. However, the tools available for mechanoreflex study were limiting. Gadolinium, a mechanically-activated

channel inhibitor, was the primary tool used to study the mechanoreflex. It was first used in 1988 by Millet and Pickard (35) as a blocker of mechanically-activated channels in plants. Its utility in mammalian physiology was quickly recognized, making it one of the leading ways to study mechanotransduction. However, its experimental applications come with some serious considerations (8). Difficulty arises in using gadolinium to isolate the physiological response from mechanically-activated channels. False negatives may arise due the insensitivity of certain channels to gadolinium (44, 49) or to the presence of certain anions that readily bind to gadolinium ions and render them functionally inactive (14, 32). This latter consideration makes it particularly difficult to determine the amount of the drug that is active within a given experiment. Further, false positives may arise due to the non-specific nature of gadolinium (18). At similar concentrations used to block mechanically-gated channels, gadolinium has been shown to also block a host of other channels including TREK-1 (31), TRAAK (31), ASIC (3), Ca^{2+}-activated Cl^- (48), Na^+ (13), K^+ (23), P2X (39), L-type (44), N-type (6), and T-type Ca^{2+} channels (5). Thus, using gadolinium alone, it is difficult to draw definitive conclusions about the contribution of mechanically-activated channels. However, GsMTx4 has recently been identified as a potent inhibitor of mechanically-activated channels. In contrast to the blanket actions of gadolinium, GsMTx4 shows relative selectivity for piezo channels (4). Despite its ability to block certain other mechanically-activated channels, the concentrations required to block these channels do not appear to overlap with experimental doses for piezo inhibition (1, 7, 27). Thus, GsMTx4 serves as a more powerful and refined tool to examine the molecular basis of mechanoreflex activation.

Data on the effect of gadolinium on the time course of the pressor response during stretch and/or contraction in is relatively absent from the literature, with one notable exception.

Specifically, Hayes at al. (19) reported that gadolinium significantly reduced the pressor response throughout the duration of both a dynamic contraction and static stretch maneuver in decerebrate cats. Because gadolinium is a non-selective blocker of mechanically-activated channels, it most likely blocked both rapidly- and slowly-deactivating channels during both of the maneuvers (19). Other studies only report that gadolinium reduced the peak pressor response during static contraction and static stretch (37, 46) but do not report pressor time course data.

We were intrigued by the marked differences in the effect of GsMTx4 on the time course of the pressor response during dynamic (present investigation) compared to static (9) hindlimb skeletal muscle stretch (Figure 5). During dynamic stretch, the effect of GsMTx4 was significant throughout the duration of the maneuver (Figure 5C). In contrast, during static stretch, GsMTx4 initially showed a large effect; however, as the stretch progressed beyond the first five seconds, the effect was largely diminished and no longer reached statistical significance (Figure 5D). We speculate that this is due to differential stimulation of mechanically-activated channels between static and dynamic stretch. Interestingly, piezo 2 channels have been observed to exhibit rapid deactivation kinetics. In 2010, Coste et al. (11) found that DRG neurons transfected with piezo 2 siRNA showed a 75% reduction in currents with inactivation kinetics (τ_{inac}) less than 10ms compared to control DRG neurons; whereas currents with 10ms<τ_{inac}<30ms and τ_{inac}>30ms were unaffected in cells transfected with piezo 2 siRNA. Further, following siRNA transfection, the proportion of non-responding neurons increased, corroborating the idea that rapidly deactivating neurons rely on piezo 2 channels for their response. Another study (40) used gene deletion to eliminate piezo 2 expression, leading to near elimination of rapidly-deactivating currents. Based on these rapid deactivation kinetics, we believe that the repetitive mechanical stimulus present during the dynamic stretch protocol likewise results in repetitive activation of piezo2 channels,

whereas static stretch provides only an initial stimulus to piezo 2 channels. In short, piezo 2 channels likely mediate the pressor response throughout the duration of a dynamic stretch protocol, whereas during a static stretch protocol, they mediate the early but not the sustained pressor response.

Future directions for this research may include investigation of channels with slowly-deactivating currents. The possibility exists that channels with slow kinetics may play a larger role in evoking the response to static stretch compared to dynamic stretch simply because their response does not necessitate repetitive mechanical stimulus. This research may focus on channels such as tentonin 3, which was recently identified as essential for slowly-adapting currents in DRG neurons (22). Further, annexin A6, a membrane-associated calcium binding protein, was recently shown to be involved in regulation of slowly-deactivating currents in sensory neurons (41). In contrast to the actions of GsMTx4, it is possible that blockade of slowly-deactivating channels, such as those previously mentioned, may block the sustained pressor response, but not the early response during static stretch.

There are three potential limitations to our current study. First, because GsMTx4 is only partially selective for piezo 2 channels, we are unable to make statements about piezo 2 activation with definite certainty. GsMTx4 is also known to block various other molecular channels including TRPC1, TRPC6, and TRPV4; however, the concentrations of GsMTx4 required to block those channels is 8 to 10 times higher than the calculated concentration of GsMTx4 within the hindlimb circulation in our experiments (1, 7, 10, 27). Further, GsMTx4 does not differentiate between blocking piezo 1 and piezo 2 channels; however, piezo 2 channels are present to a much greater extent on sensory neurons (9) and have a much more defined somatosensory role. Although GsMTx4 is a large, lipophilic molecule, Hotta et al. demonstrated

that molecules ≤10 kDa extravasate rapidly from the skeletal muscle microcirculation (24). This suggests that GsMTx4 was able to diffuse out of the circulation into the skeletal muscle interstitial space where it could act on the sensory endings of the muscle afferents. Based on these data, our experimental results were likely due to the blockade of piezo 2 channels. Second, while our data is consistent with the assumption that dynamic stretch did not produce any metabolites which stimulated the muscle afferents, the possibility remains that dynamic stretch may produce a substance which sensitizes muscle afferents. Third, previously reported data in cats indicates that only 40-50% of the afferents that respond to tendon stretch also respond to contraction and vice versa (20). If this had occurred in our experiments, there would be limited application of our data as an isolation of the mechanical stimulus present during exercise. However, Stone et al. (47) reported in freely perfused rats that 87% of the afferents that responded to tendon stretch also responded to contraction. Based on this data, muscle stretch appears to activate a very similar population of afferents compared to contraction. Thus, in rats, using stretch provides valuable insight into the mechanical stimulus present during contraction.

In conclusion, we found that GsMTx4 reduced the pressor response throughout the duration of a 30 second dynamic stretch protocol. This finding stands in contrast to its actions during static stretch, which were primarily confined to the initial five seconds of the maneuver (9). Differential stimulation of metaboreceptors between static and dynamic contraction has long been established; however, our current findings present a corollary to this. Much like static and dynamic contraction produce metabolic signals that stimulate different metabolically-activated receptors, static and dynamic stretch appear to stimulate different mechanically- activated channels. Because the mechanoreflex is often exaggerated in disease conditions (10, 28, 29, 33,

34, 45, 46), the present study yields valuable insight into the mechanism of mechanoreflex activation, which may be used in the development of future therapeutic modalities.

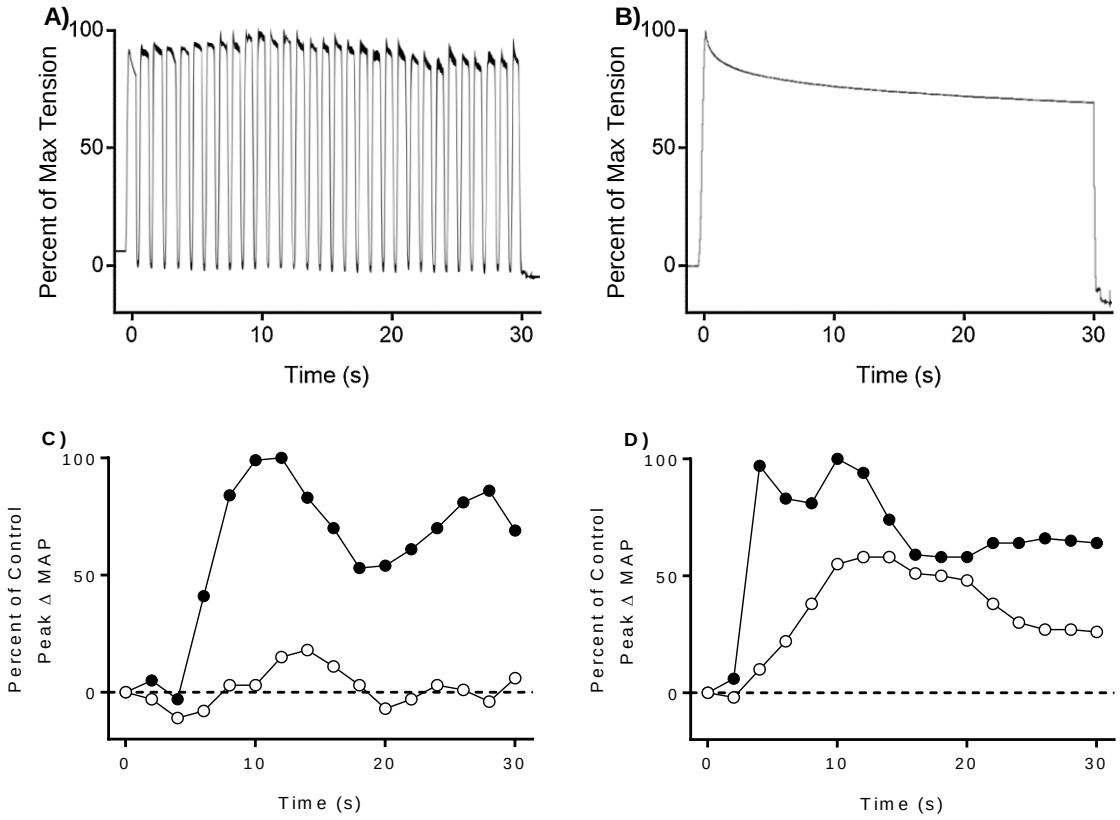

Figure 5. Comparison of the effect of GsMTx4 on the MAP response during dynamic and static hindlimb muscle stretch

Representative tension tracings of dynamic (A) and static (B) stretch compared with their respective mean pressor responses (C, D). Pressor responses are plotted as a percent of the maximum change in MAP during the control condition. Panel D is a reanalysis of data previously published by Copp et al. (9). Note that the effect of GsMTx4 on the pressor response to static stretch is confined to the first ~5 seconds of the maneuver whereas GsMTx4 reduced the pressor response throughout the duration of a dynamic stretch maneuver.

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