Mechanistic bases of the exaggerated mechanoreflex in peripheral artery disease

by

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B.S., Kansas State University, 2015
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Abstract

Mechanically-activated (MA) channels located on group III and IV skeletal muscle afferents stimulated during contraction elicit necessary autonomic and cardiovascular adjustments to exercise. However, in peripheral artery disease (PAD) MA channels become chronically sensitized and contribute to exaggerated mechanoreflex activation. Exaggerated mechanoreflex activation results in aberrant increases in sympathetic nervous system activity, heart rate, and blood pressure during exercise. This is of clinical importance because acute exaggerated autonomic and cardiovascular responses during exercise increase the patient's risk of suffering from an adverse cardiovascular event. Products of cyclooxygenase enzyme activity have been shown to chronically sensitize the mechanoreflex in PAD but the identity of the muscle afferent receptors that mediated the sensitization remained unclear. In this sequence of experiments presented in my dissertation, we use a rat model of simulated PAD in which the femoral artery is chronically ~72 hrs ligated which mimics the blood flow patterns and the exaggerated blood pressure response during exercise in PAD patients. To gain insights into exaggerated mechanoreflex activation, we use a 1 Hz repetitive/dynamic hindlimb skeletal muscle stretch (a model of mechanoreflex activation isolated from contraction-induced metabolite production) and dynamic hindlimb skeletal muscle contraction to explore downstream intracellular signaling pathways and structural elements associated with MA channel gating within sensory neurons. In the studies presented below, we find that both biochemical and biophysical properties of sensory neurons contribute to MA channel sensitivity and the exaggerated mechanoreflex in PAD. In the first study (Ch. 2) we found that thromboxane A_2 (TxA_2) receptor blockade reduced the mechanoreflex in rats with ligated femoral arteries. TxA_2 receptors are coupled to G_q proteins that when stimulated active phospholipase C which cleaves phosphatidylinositol 4,5-bisphosphate to form diacylglycerol and inositol-1,4,5 trisphosphate (IP_3). IP_3 stimulates its receptor on the endoplasmic reticulum to cause calcium release into the cytosol. Elevated cytosolic calcium has been shown to sensitize MA piezo channels, the channel thought to underlie mechanoreflex activation. In the second study (Ch. 3) we found the IP_3 receptor blockade reduced the mechanoreflex in rats with ligated femoral arteries. Intracellular signaling can alter structural components of the cell including the cytoskeleton. Actin is one of the three major components of the cytoskeleton and during inflammatory conditions, there is

increased actin polymerization (i.e., more F-actin production). Increased F-actin causes a change in static plasma membrane tension which increases the sensitivity of MA piezo channels. In the third study (Ch. 4) we found that actin polymerization inhibition reduced the exaggerated mechanoreflex in rats with ligated femoral arteries. The culmination of these studies provides evidence of biochemical and biophysical components of MA channel modulation that result in chronic sensitization and an exaggerated mechanoreflex in a rat model of simulated PAD.

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Approved by:
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Preface

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Rollins, K. S., Butenas, A. L., Felice, K. P., Matney, J. E., Williams, A. C., Kleweno, T. E., & Copp, S. W. (2020). Thromboxane A2 receptors mediate chronic mechanoreflex sensitization in a rat model of simulated peripheral artery disease. *American Journal of Physiology-Heart and Circulatory Physiology*, *319*(2), H320-H330.

Chapter 1 - Introduction

Revolutionary scientists in the late 1800s and early 1900s discovered that increases in heart rate and blood pressure during exercise occur by both central and peripheral mechanisms (2, 36, 42). Central command is a feedforward, anticipatory system that rapidly activates sympathetic activity at the onset of exercise (2, 24, 29, 42). The exercise pressor reflex (EPR) is a feedback mechanism activated by sensory endings of thinly myelinated group III and unmyelinated IV afferents in the skeletal muscle (15, 53, 57, 58). Group III and IV thin fiber muscle afferents are activated by mechanical deformation (i.e., mechanoreflex) and by metabolic stimuli, (i.e., metaboreflex). Those feedback signals synapse onto spinal interneurons which transmit signals to the regions of the brainstem responsible for increasing sympathetic outflow to facilitate skeletal muscle perfusion during exercise (4, 25, 53, 62, 65). While both the mechanoreflex and the metaboreflex are individually being advanced in their understanding, they are advancing at vastly different rates. The goal of this review is to expand on the nuances that emerged during the methodological separation of the mechanoreflex from the metaboreflex in animals, additionally highlighting specific mechanoreceptors/mechanically-activated (MA) channels that appear to play a role in the activation of the mechanoreflex.

Since the seminal studies of Alam and Smirk (2), a keystone of our original understanding is that contracting muscles become ischemic during exercise, thus stimulating group IV “metabo-sensitive” afferents causing reflex increases in sympathetic activity, heart rate, and blood pressure, which augment skeletal muscle perfusion to alleviate ischemia (68). During the late 1940s to early 1950s in which the field was advancing our mechanistic understanding of the involvement of unmyelinated afferents, considerable information was also gained about the distribution of myelinated afferents within the skeletal muscle (31, 47). Myelinated muscle afferents naturally fell into 3 different size categories, ranging from more densely-myelinated group I and IIs to thinly-myelinated group IIIs (31, 35, 47, 67), However, little attention had been paid to group IIIs because the general belief was that there were few of them within the muscle (31). It was not until 1959 when Barker et. al. (7) histologically identified “pressure” receptors that appeared to be located within the skeletal muscle of cats. Subsequently, to determine their functional role, Paintal et. al., 1960 set out to explain the behavior of group III afferents by non-noxiously stretching, squeezing, poking, prodding, and pressing cat hindlimb

skeletal muscle, concluding that a majority of group III afferent sensory endings terminated in rapidly-adapting "pressure" receptors that are activated by a variety of mechanical stimuli (63). Further investigation would be required to determine if stimulation of rapidly-adapting group III "pressure" receptors, results in autonomic activation in the vasomotor centers of the brain to increase sympathetic outflow, heart rate, and blood pressure.

Divorcing the mechanoreflex from the metaboreflex has become particularly important because metabolites produced during contraction have been shown to acutely sensitize MA channels (37). In humans, isolated mechanoreflex activation has been undertaken in a variety of ways, including passive muscle stretch (21, 22, 28), electrical muscle stimulation (14), low-intensity exercise (8, 34, 56, 59), eccentric contraction protocols (12, 23), passive exercise (61, 80), lower body negative pressure (26), and external muscle compression (13, 82). Each method of activation has its limitations, nevertheless, in their totality conclude that the mechanoreflex appears to play an important role in the relatively instantaneous increase in sympathetic activity to redistribute blood flow away from inactive vascular beds and toward the active skeletal muscle at the onset of exercise. In animals, isolating the mechanoreflex to study reflex control of heart rate and blood pressure received little attention until Stebbins et. al. 1988 developed a 30 s passive hindlimb muscle static stretch of the triceps surae muscle group in anesthetized cats (71). Static stretch was deemed a "pure" mechanical stimulus after confirming no elevation in venous blood metabolites, including potassium and lactate, as well as no changes in pH or O_2 content during the stretch maneuver. Passive stretch appeared to be a useful technique for delineating metabolic and mechanical signals present during muscle contraction, but initially raised two important questions (71).

First, will muscle contraction and stretch result in the activation of the same afferents? If stretch is to be used as a surrogate of isolated mechanoreflex activation, then the same afferents that are activated during contraction must also respond to stretch. Neural recordings of group III and IV afferents revealed 80% of all afferents units responded to both hindlimb muscle contraction and stretch in cats (55) and 87% of all group III afferents (72) responded to both hindlimb muscle contraction and stretch in rats. Static hindlimb muscle stretch in both cats and rats does appear to stimulate the majority of the MA channels on sensory afferents that are also activated during static contraction.

Second, does the magnitude and direction of the change (stretch/lengthening vs. contraction/shortening) in muscle length result in the same amount of MA channel stimulation? This question can be answered using data from neural afferent recordings and reflex cardiovascular experiments. Mense et. al. 1983, left the calcaneus tendon in cats intact and stretch movements were made by manually bending the paw with a stretch that was either static (i.e., paw as bent and kept in the bent position), or dynamic, (i.e., the paw was bent once every second), with a stretch mild enough (500-750 P) that it did not cause discomfort in awake cats. They found that all group III afferent units, except for one, responded to both a static stretch and a dynamic stretch stimulus. Moreover, they show that the time course of MA channel adaption during static stretch paralleled that of static contraction, meaning MA channels displaying rapid adaption to contraction also displayed rapid adaption during stretch. Although the changes in muscle length were not directly reported, the mild stretch used was sufficient enough to activate the same group III afferents activated by low intensity contractions (20% of max), and displayed the same discharge properties upon activation (55). Further, Adreani et. al. found that 75% of group III afferents responded to low intensity walking exercise in decerebrate cats. Group III afferent discharge was synchronous to muscle contraction, showing extreme sensitivity even to low levels of tension with no evidence of desensitization or adaptation (1, 64). Interestingly, cats produce peak muscle tension in the eccentric phase of contraction during walking exercise (30, 81). These pieces of information would suggest that MA channels activation could be occurring during eccentric contraction (*i.e.*, lengthening of the muscle fiber under tension) (1). This supports the use of passive stretch of the muscle to investigate MA channel activation present during eccentric contraction in quadrupeds. In bipeds, basic locomotor tasks require eccentric contraction (70), however, whether the eccentric phase of walking contributes to mechanoreflex activation and subsequent cardiovascular responses in humans will require further investigations. While nearly all EPR-related research is done during an isolated concentric contraction of a hindlimb (16, 17, 38-41, 55, 79), we must also acknowledge that MA channel activation could occur during eccentric contraction (12, 23).

Additionally, Stebbins et. al. 1988 used ultrasonic crystals to examine the extent of the triceps surae muscle length change when the calcaneus tendon was left intact in anesthetized cats to determine physiological length. Stretching the muscle within physiological length (*i.e.*, calcaneus was intact) was determined to generate ~2kg of tension, yet they were able to produce

significant increases in blood pressure by stretching the triceps surae muscle group as little as 500g. Additionally, they reported that when static contraction and stretch tensions were matched, the blood pressure response to stretch was about one half that during contraction (71). While static stretch provides a great tool to study MA channel activation that would be present during static contraction, we have since adopted the 30 s 1 Hz dynamic stretch protocol in our decerebrate rat preparation, originally performed in anesthetized cats, to gain insight into MA channel activation during rhythmic exercise (20, 39). Kempf. et. al. 2018 found that dynamic contraction and stretch with matched tension-time indices resulted in similar increases in renal sympathetic nervous system activity and blood pressure, suggesting that MA channels activated during dynamic stretch mediated almost the entirety of the pressor response during contraction in decerebrate rats (39). Differences in MA channel stimulation (static vs dynamic) could account for differences in the pressor response. Since the dynamic stretch protocol was established to isolate the MA channel stimulation in the absence of acute contraction-induced metabolic production, it has been used by our laboratory and will be seen throughout the studies presented in this dissertation to study the mechanisms behind mechanoreflex activation in regards to neural control of the circulation in both healthy rats and rat models of cardiovascular disease.

Identifying specific receptors that are directly activated by mechanical deformation has proved challenging due to minimal pharmacological agents that are selective for MA channels. The commonly used trivalent lanthanide, gadolinium has been shown to reduce group III afferent responsiveness as well as the blood pressure response to hindlimb muscle stretch in cats (32, 33, 52). However, false positive findings are of concern because gadolinium is not a selective antagonist for MA channels. It has been used to block piezo currents, a novel class of recently discovered MA channel (19), but also has off-target effects including inhibition of L-type, T-type, and N-type Ca^{2+}, Na^{2+}, K^{+} (TREK-1 and TRAAK, (51)), and Ca^{2+}-activated Cl^{-} channels (11). It wasn't until 2000 when a peptide isolated from tarantula venom, GsMTx4, was shown to act as a gating modifier by inserting into the lipid bilayer, forcing MA piezo channels (3, 6) into their closed state, thus being used as a more selective cationic MA channel inhibitor (9, 74). In rats, GsMTx4 has been shown to reduce both the EPR during dynamic contraction (16) as well as the mechanoreflex during static and dynamic hindlimb stretch (16, 69). GsMTx4 was found to inhibit the pressor response to the first ~5 s of the 30 s static stretch protocol when tension is being developed, but has no effect on the sustained phase when tension remained constant (16).

However, GsMTx4 reduced the pressor response throughout the duration of the dynamic stretch protocol when muscle length was constantly changing (69). Collectively, these findings suggest that rapidly-adapting piezo channels are, in part, responsible for mechanoreflex activation in rats.

The mechanoreflex is necessary for appropriate cardiovascular responses to exercise in health, but contributes to sympathoexcitation and exaggerated increases in blood pressure in cardiovascular disease. A significant body of literature demonstrates that peripheral artery diease (PAD) patients experience sympathoexcitation and exaggerated increases in blood pressure during multiple forms of exercise compared to age-matched healthy counterparts (5, 48, 60) and this exaggeration is, in part, due to an exaggerated mechanoreflex (60). Specifically related to the following studies presented in the dissertation, we use a rat model of peripheral artery insufficiency in which the femoral artery is ligated ~72 hr before the start of an experiment (66). This model results in an exaggerated increase in sympathetic activity, heart rate, and blood pressure response during treadmill walking, static and dynamic hindlimb contraction, and dynamic hindlimb stretch (39, 43, 44, 75, 88) and has been used in many studies with the goal of identifying mechanisms of EPR exaggeration and mechanoreflex sensitization (10, 17, 18, 27, 43-46, 49, 50, 54, 72, 73, 75-78, 83-87, 89). Understanding the underlying mechanisms of the exaggerated sympathetic nervous system activity and blood pressure response during exercise is of clinical importance because acute exaggerated increases in sympathetic nervous system activity and blood pressure result in an increased risk of suffering an ischemic event such as fibrillation or stroke.

In summary, the studies in this dissertation will use dynamic hindlimb muscle stretch as a vital tool to investigate the mechanoreflex in isolation from acute contraction-induced metabolite production. This protocol has become especially important in mechanistic animal studies aimed at identifying underlying origins of the exaggerated mechanoreflex seen in patients with multiple forms of cardiovascular disease.

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Chapter 2 - Thromboxane A_2 receptors Mediate Chronic Mechanoreflex Sensitization in a Rat Model of Simulated Peripheral Artery Disease

Abstract

The exercise pressor reflex is a feedback autonomic and cardiovascular control mechanism evoked by mechanical and metabolic signals within contracting skeletal muscles. The mechanically sensitive component of the reflex (the mechanoreflex) is exaggerated in peripheral artery disease (PAD) patients and in a rat model of simulated PAD in which a femoral artery is chronically ligated. Products of cyclooxygenase enzyme activity have been shown to chronically sensitize the mechanoreflex in PAD but the identity of the muscle afferent receptors that mediate the sensitization is unclear. We hypothesized that injection of the endoperoxide 4 receptor (EP4-R) antagonist L161982 or the thromboxane A_2 receptor (TxA_2-R) antagonist daltroban into the arterial supply of the hindlimb would reduce the pressor response to repetitive, dynamic hindlimb skeletal muscle stretch (a model of isolated mechanoreflex activation) in rats with a femoral artery that was ligated ~72 hours before the experiment but not in rats with freely perfused femoral arteries. We found that EP4-R blockade had no effect on the pressor response (peak Δ mean arterial pressure) to stretch in "freely perfused" (n=6, pre: 14±2, post: 15±2 mmHg, p=0.97) or "ligated" (n=8, pre: 29±4, post: 29±6 mmHg, p=0.98) rats. In contrast, TxA_2-R blockade had no effect on the pressor response to stretch in freely perfused rats (n=6, pre: 16±3, post: 17±4 mmHg, p=0.99) but significantly reduced the response in ligated rats (n=11, pre: 29±4, post: 17±5 mmHg, $p<0.01$). We conclude that TxA_2-Rs contribute to chronic mechanoreflex sensitization in the chronic femoral artery ligated rat model of simulated PAD.

Introduction

The exercise pressor reflex (EPR) is a feedback autonomic and cardiovascular control mechanism that arises within contracting skeletal muscles and contributes importantly to increased sympathetic nervous system activity during exercise (1, 3, 21, 23, 37, 41, 59). The afferent arm of the EPR is comprised of thinly-myelinated group III and unmyelinated group IV muscle afferents whose sensory endings are stimulated primarily by mechanical and metabolic signals, respectively (22-24, 37-39, 51). The central projections of the group III and IV afferents, collectively termed thin fiber muscle afferents, synapse onto spinal interneurons which project to autonomic control circuits within the medulla of the brainstem (4, 20, 29-31, 41, 50). In healthy individuals, the EPR redistributes cardiac output and increases arterial blood pressure, which increases blood flow to contracting muscles and supports exercise performance (2, 3, 46, 59).

A significant body of literature indicates that the EPR is exaggerated in peripheral artery disease (PAD) patients (5, 15, 34, 40, 43) and in a rat model of simulated PAD in which a femoral artery is ligated ~72 hrs before the experiment (e.g., 11, 58, 60). An exaggerated EPR in PAD patients contributes to the augmented sympathetic nervous system and blood pressure reactivity present during exercise that is observed in this clinical population (6, 7, 15, 43, 44). Investigation of the mechanisms of the exaggerated EPR in PAD is important because augmented sympathetic nervous system and blood pressure reactivity during exercise contributes to exercise intolerance and increases the risk of an ischemic cardiovascular event such as cardiac fibrillation and/or stroke (13, 32, 49).

Empirical evidence in PAD patients and in the femoral artery ligated rat model of simulated PAD indicates that the mechanically-activated portion of the EPR (i.e., the mechanoreflex) contributes importantly to the overall EPR exaggeration (10, 33, 43, 44). Theoretically, aberrant mechanosensation could result from increased expression and/or sensitization of mechanically activated channels on the sensory endings of thin fiber muscle afferents. Regarding the first mechanism, Copp et.al (10) found recently that chronic femoral artery ligation in the rat did not impact protein expression of mechanically-activated piezo channels in lumbar dorsal root ganglia (DRG). Despite not having a complete picture of the proteins that underlie mechanoreflex activation, that initial evidence suggests that increased expression of mechanically activated channels does not contribute to the exaggerated mechanoreflex in PAD. Regarding the second mechanism, our laboratory has used a 30 second,

1 Hz dynamic/repetitive rat hindlimb muscle stretch protocol as a model of isolated mechanoreflex activation (8, 25, 47, 52). We found recently that the pressor and sympathetic nerve responses evoked during stretch of a rat hindlimb with a ligated femoral artery were greater than those found during stretch of the contralateral freely perfused hindlimb (25). That finding suggested a femoral artery ligation-induced "chronic" sensitization of mechanically activated channels that did not depend on acute increases in metabolite production during skeletal muscle contraction. Moreover, cyclooxygenase (COX) inhibition within the hindlimb reduced the pressor response to skeletal muscle stretch in "ligated" but not "freely perfused" rat hindlimb muscles (8). Those findings indicate that the chronic sensitization of mechanically activated channels is attributable to some aspect of COX enzyme activity. Interestingly, femoral artery ligation had no effect on skeletal muscle COX protein expression or activity, but increased lumbar DRG protein expression of endoperoxide 4 receptors (EP4-R) and thromboxane A2 receptors (TxA_2-R) (8); receptors associated primarily with the COX products prostaglandin E2 and TxA_2, respectively (8, 62). Thus, chronic sensitization of the mechanoreflex appears to occur through EP4-R and/or TxA_2-R-dependent mechanisms, but the specific contributions of the different receptor classes during dynamic mechanoreflex activation is unknown.

Based on the information above, we performed the present investigation to determine the roles played by EP4-R and TxA_2-R in the chronic sensitization of the mechanoreflex present in the rat model of simulated PAD in which a femoral artery is ligated ~72 hrs before the experiment. Specifically, we tested the hypothesis that the inhibition of EP4-R and TxA_2-R on the sensory endings of the thin fiber muscle afferents would reduce the exaggerated pressor response evoked during 30 seconds of 1 Hz dynamic hindlimb muscle stretch in rats with a previously ligated femoral artery, but not in rats with freely perfused femoral arteries.

Methods and Materials

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 (~12-15 weeks old) male Sprague-Dawley rats (n=57, average body weight: 416±10 g; Charles River Laboratories). The rats were housed two per cage in temperature (maintained at 22°C) and light (12-12 hr light-dark cycle)-controlled accredited facilities with standard rat chow and water provided ad libitum. At the end of each experiment, the decerebrated rats (see below) were killed by an intravenous injection of saturated (>3 mg/kg) potassium chloride.

Survival surgical procedures. Forty-five rats in this investigation had their left femoral artery ligated ~72 hr before the terminal experimental protocol was performed. Briefly, rats were anesthetized with 3% isoflurane (balance O_2) and their left femoral artery was surgically exposed and ligated tightly with 5-0 silk suture ~3–5 mm distal to the inguinal ligament. In six additional rats, a sham ligation procedure was performed in which the left femoral artery was surgically exposed, and 5-0 suture was passed under the femoral artery but not tied. In both "ligated" and "sham" rats, the incisions were closed, and the rats were administered meloxicam (1 to 2 mg/kg s.c.) as an analgesic.

Surgical procedures for experiments: On the day of the experiment, rats were anesthetized with ~3% isoflurane (balance O_2). Adequate depth of anesthesia was confirmed by the absence of toe-pinch and blink reflexes. The trachea was cannulated, and the lungs were mechanically ventilated (Harvard Apparatus model 683,) with the gaseous anesthetic (2% isoflurane balance O_2) until the decerebration was completed (see below). In all rats, the right jugular vein and both common carotid arteries were cannulated with PE-50 catheters for the administration of fluids/drugs, measurement of arterial blood pressure (Physiological Pressure Transducer, AD Instruments), and sampling of arterial blood gases (ABL80 Flex, Radiometer). Heart rate (HR) was measured by electrocardiogram. In all rats, the left calcaneus bone was severed and the triceps surae (gastrocnemius, soleus and plantaris) muscles were exposed by reflecting the overlying skin and skeletal muscles. A string was then tied to the distal Achilles tendon and severed calcaneus, which linked the triceps surae muscles to a force transducer (Grass FT03) and rack and pinion that could be manually turned. In the six sham rats, six non-

operated rats, and 33 ligated rats, the left superficial epigastric artery was cannulated with a PE-8 catheter whose tip was located near the junction of the superficial epigastric artery and the femoral artery. In the rats in which a catheter was placed in the left superficial epigastric artery, a reversible snare was placed around the left iliac artery and vein (i.e., proximal to the location of the catheter placed in the superficial epigastric artery). In four additional ligated rats, the right superficial epigastric artery was cannulated with a PE-8 catheter and a reversible snare was placed around the right iliac artery and vein.

After the initial surgical procedures, all rats were placed in a Kopf stereotaxic frame with clamps placed around the pelvis. Dexamethasone (0.2 mg i.v.) was injected to minimize brainstem edema. A precollicular decerebration was performed and all neural tissue rostral to the superior colliculus was aspirated. After the decerebration was completed, anesthesia was terminated and the lungs were mechanically ventilated with room air. The decerebration procedure was performed because anesthesia has been shown to depress the exercise pressor reflex in the rat (53). Arterial blood gases and pH were measured periodically with a blood gas analyzer and maintained within normal limits ($PaCO_2$: 35–45 mmHg, PaO_2: ~100 mmHg, pH: 7.35–7.45) by adjusting ventilation and/or administering intravenous sodium bicarbonate (8.5%). Core temperature was measured by a rectal probe and maintained at ~37-38°C by an automated heating system (Harvard Apparatus) and heat lamp. For all rats in which dynamic stretch was performed, the paralytic pancuronium bromide (1 mg/kg i.v.) was injected prior to the initiation of any stretch maneuver in order to prevent any spontaneous or reflex muscle contraction which would produce a metabolic stimulus.

Primary mechanoreflex experimental protocols (Fig. 1). In three sham rats, three non-operated rats (i.e., “freely perfused), and eight ligated rats, we compared the pressor and cardioaccelerator response to dynamic hindlimb muscle stretch before and after the injection of the EP4-R antagonist L161982 (1 μg dissolved in 0.2 ml of 0.15% DMSO) into the arterial supply of the hindlimb through the superficial epigastric artery catheter. In different groups of three sham rats, three freely perfused rats, and seven ligated rats, we compared the pressor and cardioaccelerator response to dynamic hindlimb muscle stretch before and after the injection of the TxA_2-R antagonist daltroban (80 μg dissolved in 0.4 ml of 1% DMSO) into the arterial supply of the hindlimb through the superficial epigastric artery catheter. The control (i.e., pre-EP4-R or TxA_2-R blockade) stretch was performed at least 60 minutes following termination of

isoflurane anesthesia. Specifically, to begin each protocol, baseline muscle tension was set at ~0.08 to 0.1 kg and baseline data were collected for 30 s. A 30 s control dynamic stretch maneuver was then performed by an experienced investigator who manually turned the rack and pinion in a rhythmic fashion at a 1 Hz frequency with the aid of a metronome. The investigator aimed to develop ~0.6 to 0.8 kg of tension during each dynamic stretch maneuver because that is the tension typically developed during hindlimb muscle contractions in decerebrate rat preparations (9, 10, 25). Moreover, the investigator aimed for consistent levels of tension development across individual stretches within each 30 s maneuver although slight variability in tension development was often present (see Figs. 3 and 5). The dynamic stretch protocol was adapted from protocols described by Stebbins et al. (55) and Daniels et al. (12) in the cat. After the control stretch maneuver, the snare on the left iliac artery and vein was tightened and either L161982 or daltroban was injected into the hindlimb arterial supply. For the EP4-R experiments, five minutes after L161982 injection, the snare was released and the hindlimb was reperfused for 25 minutes before the post-EP4-R blockade stretch was performed. For the TxA_2-R experiments, five minutes after daltroban injection, the snare was released and the hindlimb was re-perfused for 10 minutes before the post-TxA_2-R blockade stretch was performed. The dose of the antagonists and timing of the post-antagonist stretch maneuvers was based on recent investigations (27, 62). Tightening the iliac artery/vein snare in the manner described at least partially trapped the drug within the arterial supply of the hindlimb and limited its systemic circulation.

In an additional group of four ligated rats, we investigated the effect of TxA_2-R blockade with daltroban (80 μg) dissolved in 100 mM sodium carbonate on the pressor and cardioaccelerator response to dynamic hindlimb muscle stretch (see Results for more details). The injection of daltroban and timing of the post-TxA_2-R blockade stretch maneuver was performed exactly as described above.

Vehicle control experiments: In six ligated rats, we investigated the effect of 1% DMSO (vehicle for the TxA_2-R antagonist daltroban) on the pressor and cardioaccelerator response to dynamic hindlimb muscle stretch. These experiments were performed exactly as described above for the daltroban experiments except 0.4 ml of 1% DMSO alone was injected into the arterial supply of the hindlimb via the left superficial epigastric artery catheter.

EP4-R blockade efficacy control experiments: In three ligated rats, we attempted to confirm the efficacy of EP4-R blockade by reproducing the previous finding that L161982 (1 μg) reduced the pressor and cardioaccelerator response to static hindlimb muscle contraction in ligated rats (62). To elicit static contraction, the sciatic nerve was exposed and stimulated at a voltage of 1.5-2x motor threshold, a pulse duration of 0.01 ms and a frequency of 40 Hz for 30 s. The dose of L161982 (1 μg) and timing of contractions relative to L161982 injection into the arterial supply of the hindlimb via the left superficial epigastric artery catheter was performed exactly as described above for the mechanoreflex protocol. At the end of each experiment, to ensure that the increase in blood pressure during contraction was not due to electrical activation of the axons of the thin fiber afferents in the sciatic nerve, we administered the paralytic pancuronium bromide (1 mg/kg i.v.) and the sciatic nerve was stimulated for 30 s with the same parameters as those used to elicit contraction during the experiment. No increase in blood pressure was observed during the stimulation period after the administration of pancuronium bromide in any contraction experiment confirming that the increases in blood pressure observed during hindlimb muscle contraction were reflex in nature.

Contralateral systemic control experiments: In four ligated rats, we investigated the effect of daltroban injection into the arterial supply of the freely perfused right hindlimb on the pressor and cardioaccelerator response to dynamic stretch of the triceps surae muscles of the left ligated hindlimb. These experiments served as a control for the possibility that the effect of daltroban on the mechanoreflex produced when it was injected into the arterial supply of the left hindlimb in ligated rats may have been due to a systemic effect rather than a local effect. After the control stretch maneuver, daltroban was injected into the arterial supply of the right hindlimb through the right superficial epigastric artery catheter similar to the method described above. Stretch of the left hindlimb muscles was then performed with the same timing relative to drug injection as described above.

"Off-target" effects of daltroban control experiments: In five ligated rats, we investigated the effect of daltroban injection into the arterial supply of the left hindlimb on the pressor and cardioaccelerator response evoked in response to the hindlimb arterial injection of lactic acid (24 mM, 0.2 ml). Following the control injection of lactic acid into the arterial supply of the left hindlimb through the superficial epigastric artery catheter, the injection of daltroban into the arterial supply of the hindlimb was performed exactly as described above for the primary

mechanoreflex experiments which was then followed by a second lactic acid injection. In a subgroup of three of these ligated rats, we found that 0.2 ml of saline alone rapidly injected into the arterial supply of the hindlimb via the superficial epigastric artery did not produce any increase in blood pressure or heart rate.

At the end of each experiment described above in which an injection into the arterial supply of the hindlimb through the superficial epigastric artery catheter was performed, Evans blue dye was injected into the catheter in the same manner as the drug to confirm that the injectate had access to the triceps surae muscle circulation. The triceps surae muscles were observed to stain blue in all experiments.

Cyclic adenosine monophosphate (cAMP) assay: In 10 rats, we measured cAMP concentration in L4 and L5 DRG innervating a hindlimb whose femoral artery was ligated and from the DRG innervating the contralateral hindlimb whose femoral artery was freely perfused using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. Briefly, DRG were homogenized in the sample diluent provided according to manufacturer's instructions (Arbor Assay). Provided standards were measured in triplicate and experimental samples were measured in duplicate. The average coefficient of determination (r^2) of the standard curves across all assays was 0.99. The average coefficient of variation across all samples and standards was 3.6±0.6% with a range of 0.2 to 19.1%. All samples fell within the measurement range (0.617-150 pmol/ml) of the ELISA kit.

Data analysis. Data were collected with a PowerLab and LabChart data acquisition system (AD Instruments). Arterial blood pressure, electrocardiogram, and muscle tension were measured, and mean arterial pressure (MAP) and heart rate (HR) were calculated and displayed in real time and recorded for offline analysis. Baseline MAP and HR were determined from the 30 s baseline periods that preceded each maneuver. The peak pressor (peak Δ MAP) and cardioaccelerator (peak Δ HR) responses were calculated as the difference between the peak values wherever they occurred during the 30 second maneuvers and their corresponding baseline value. The tension-time indexes (TTIs) and blood pressure indexes (BPIs) were calculated by integrating the area under the data signal during the stretch or contraction maneuver and subtracting the integrated area under the data signal during the baseline period. All values are expressed as mean±SEM. Data were analyzed with paired or unpaired Student's t-tests or Sidak multiple comparisons tests as appropriate. Statistical significance was accepted at $p \leq 0.05$.

Results

Effect of sham and femoral artery ligation procedures on the mechanoreflex: Pooled data from the control condition across experimental groups indicated that the sham femoral artery ligation procedure had no effect on the pressor (peak Δ MAP sham: 12±1, non-sham freely perfused: 18±3 mmHg, p=0.16, n=6 each) or cardioaccelerator (peak Δ HR sham: 9±2, non-sham freely perfused: 6 ±2 bpm, p=0.30) response to dynamic stretch. The TTIs of the stretch maneuvers were not different between groups (sham: 13±1, non-sham freely perfused: 11±1 kg·s, p=0.09). Given that finding, the "freely perfused" groups below for the EP4-R and TxA_2-R experiments reflect a cohort of sham (n=3) and non-sham (n=3) freely perfused rats.

Similar to our recent findings (8, 25), pooled data from the current study (29 ligated rats and the cohort of 12 freely perfused rats) indicated that femoral artery ligation exaggerated the pressor (peak Δ MAP freely perfused: 15±2, ligated: 28±2 mmHg, p<0.01) and cardioaccelerator (peak Δ HR freely perfused: 8±1, ligated: 14±2 bpm, p=0.02) response to dynamic stretch. The TTIs of the dynamic stretch maneuvers were not different between groups (freely perfused: 12±1 and ligated: 13±1 kg·s, p=0.12).

Effect of EP4-R blockade on the mechanoreflex: In freely perfused (n=6) and ligated rats (n=8), we found that EP4-R blockade had no effect on the peak Δ MAP, BPI, or peak Δ HR response to dynamic stretch (Fig. 2). The TTI of the dynamic stretch maneuvers were not different between conditions in freely perfused or ligated rats. Baseline MAP and HR were not different between control and EP4-R blockade conditions in freely perfused or ligated rats (Table 1). Figure 3 shows an original tracing of the pressor and cardioaccelerator response to dynamic stretch before and after the injection of the EP4-R antagonist L161982 into the arterial supply of the hindlimb in a ligated rat.

Effect of TxA_2-R blockade on the mechanoreflex: In freely perfused rats (n=6), we found that TxA_2-R blockade with daltroban in 1% DMSO had no effect on the peak Δ MAP, BPI, or peak Δ HR response to dynamic stretch (Fig. 4). In ligated rats (n=7), we found that TxA_2-R blockade with daltroban in 1% DMSO significantly reduced the peak Δ MAP (control: 34±5, TxA_2-R blockade: 21±7 mmHg, p<0.01) and tended to reduce the peak Δ HR (control: 17±5, TxA_2-R blockade: 12±4 bpm, p=0.07) response to stretch (Fig. 4, and see also the original tracing in Fig. 5). In another group of ligated rats (n=6), we found that 1% DMSO alone had no effect on the peak Δ MAP, BPI, or peak Δ HR response to stretch (Fig. 6). The results of the

vehicle control experiments, on average, support the conclusion that 1% DMSO was not responsible for producing the effects observed when daltroban was injected into the arterial supply of the hindlimb. However, as evidenced by the individual responses in Fig. 6, we observed a large amount of variability in those vehicle control experiments. Thus, to provide even further support of our conclusions, in four additional ligated rats we performed TxA_2-R blockade experiments in which daltroban was dissolved in 100 mM sodium carbonate, a vehicle which we have found recently does not impact the pressor response to dynamic stretch in ligated rats (8). Similar to the daltroban in 1% DMSO experiments, we found that daltroban in 100 mM sodium carbonate reduced the pressor (control: 19±2, TxA_2-R blockade: 11±1 mmHg, $p<0.01$) and cardioaccelerator (control: 11±2, TxA_2-R blockade: 7±2 bpm, $p<0.01$) response to dynamic stretch. For simplicity and clarity of presentation, all experiments in ligated rats in which daltroban was injected into the arterial supply of the hindlimb (n=11 total) are presented as a cohort in Figure 4. Specifically, we found that daltroban significantly reduced the peak Δ MAP, BPI, and peak Δ HR response to dynamic stretch in the cohort of ligated rats. The TTIs of the dynamic stretch maneuvers were not different between conditions for any of the experiments described above. Likewise, baseline MAP and HR were not different between conditions for any of the experiments described above (Table 1).

EP4-R blockade efficacy control experiments: Given the lack of effect of EP4-R blockade in ligated rats described above, we performed experiments to determine if the dose of the EP4-R antagonist L161982 effectively blocked EP4-Rs on the sensory endings of the thin fiber muscle afferents innervating the triceps surae muscles. Consistent with the finding of Yamauchi et. al (62), in three ligated rats we found that EP4-R blockade with L161982 reduced the peak Δ MAP (control: 29±5, EP4-R blockade: 14±1 mmHg, $p=0.04$) response to static hindlimb muscle contraction. The TTIs of the static contraction maneuvers were not different between control (19±2 kg·s) and EP4-R blockade conditions (19±1 kg·s, $p=0.60$). Baseline MAP and HR were not different between conditions (Table 1).

Contralateral systemic control experiments: We investigated whether the effect of daltroban in ligated rats could be attributed to blockade of TxA_2-R on the sensory endings within the hindlimb musculature or, alternatively, systemic effects elsewhere in the mechanoreflex arc such as the brainstem and/or the spinal cord. In four ligated rats, we found that the injection of daltroban in 1% DMSO into the arterial supply of the contralateral right hindlimb had no effect

on the peak Δ MAP, BPI, or peak Δ HR response to dynamic stretch of the left hindlimb (Fig. 7). The TTIs of the dynamic stretch maneuvers were not different between conditions. Baseline MAP and HR were not different between the control and post-TxA_2-R blockade conditions (Table 1).

"Off-target" effects of daltroban control experiments: We investigated whether the effect of daltroban in ligated rats could be attributed to an interaction between TxA_2 receptors and mechanically-activated channels or some other local "off-target" effect such as the inhibition of voltage-gated sodium channels. In five ligated rats, we found that the injection of daltroban into the arterial supply of the ligated hindlimb had no effect on the peak Δ MAP (control: 45±7, TxA_2-R blockade: 51±4 mmHg, p=0.23) or peak Δ HR (control: 15±2, TxA2-R blockade: 15±3 bpm, p=0.84) response to arterial lactic acid injection. Baseline MAP and HR were not different between the control and post-TxA_2-R blockade conditions (Table 1).

In 10 rats, we found no difference (p=0.63) in cAMP concentration in L4 and L5 DRG innervating the hindlimb in which the femoral artery was previously ligated compared to the L4 and L5 DRG innervating the contralateral hindlimb which was freely perfused (freely perfused: 47±3, ligated: 49±4 pmol/ml).

Discussion

We investigated the role played by the COX-related EP4-R and TxA_2-R in the chronic mechanoreflex sensitization present in the rat model of simulated PAD in which a femoral artery is ligated ~72 hr before the experiment. In ligated rats, we found that TxA_2-R blockade, but not EP4-R blockade, reduced the pressor and cardioaccelerator response to dynamic hindlimb skeletal muscle stretch. In freely perfused rats, we found that EP4-R and TxA_2-R blockade had no effect on the pressor or cardioaccelerator response to dynamic stretch. The data suggest that femoral artery ligation sensitizes mechanically activated channels, at least in part, through a TxA_2 receptor-dependent mechanism. The findings enhance our understanding of the mechanisms underlying chronic mechanoreflex sensitization in this rat model of simulated PAD and, therefore, have potential implications for understanding blood pressure control in PAD patients.

Our finding that EP4-R and TxA_2-R blockade had no effect on the pressor or cardioaccelerator response to dynamic stretch in freely perfused rats is consistent with multiple studies which suggest that COX metabolites are not strictly necessary to produce reflex cardiovascular responses in healthy subjects (8, 14, 42, 44, 47). Most relevant to this investigation, in healthy/freely perfused rats, COX inhibition was found previously to have no effect on reflex cardiovascular responses during either static (42, 47) or dynamic hindlimb muscle stretch (8, 47). Moreover, Yamauchi et al. (62) found that EP4-R blockade had no effect on the pressor response to static hindlimb muscle stretch in freely perfused rats. In contrast, however, Leal et al. (27) found that TxA_2-R blockade with daltroban reduced the pressor response to static hindlimb muscle stretch in freely perfused rats. The reason for the difference between Leal et al. (27) and the present investigation is unknown but may relate to the mode of isolated mechanoreflex activation (static vs. dynamic). Nevertheless, the bulk of the available data, including those from the present investigation, indicate that neither COX enzymes nor COX metabolite receptors mediate isolated mechanoreflex activation in healthy rats.

Our finding that EP4-blockade had no effect on the pressor or cardioaccelerator response to dynamic hindlimb muscle stretch in ligated rats is an extension of the finding of Yamauchi et. al. (62) that EP4-R blockade had no effect on the reflex cardiovascular responses to static hindlimb muscle stretch in ligated rats. The possibility exists, however, that redundancy among various receptors may have masked a possible contribution of EP4-R in our experiments (56).

We used a dynamic stretch protocol in the present investigation because it replicates more closely the mechanical stimuli present when PAD patients walk than does static hindlimb muscle stretch. Moreover, we have found consistently that chronic femoral artery ligation produces an exaggerated pressor response to dynamic stretch (present investigation, 8, 25) whereas there is not consensus on whether ligation exaggerates the pressor response to static hindlimb skeletal muscle stretch (e.g., 26, 27, 33, 48).

Also consistent with the finding of Yamauchi et al. (62), we found that EP4-R blockade reduced the pressor response to static hindlimb muscle contraction. This was an important control experiment because it provided evidence that, in our hands, the injection of 1 μg of L161982 sufficiently inhibited EP4-R on the sensory endings of thin fiber hindlimb muscle afferents. We used a static rather than a dynamic hindlimb muscle contraction protocol because we needed to perform a maneuver in which EP4-Rs had been previously shown to play a role. The role played by EP4-Rs in the pressor response to dynamic skeletal muscle contractions has not been investigated and is an important future direction. Taken together, we conclude that the rat femoral artery ligation-induced upregulation of EP4-Rs (8, 62) does not contribute to the chronic sensitization of the mechanoreflex present in this simulated PAD model. EP4-Rs do, however, play a role in acute mechanoreflex sensitization and/or activation of the metabolically sensitive component of the EPR (i.e., the metaboreflex) during static rat hindlimb muscle contraction (62).

We investigated the effect of femoral artery ligation on cAMP concentration in L4 and L5 DRG to further explore the fact that an upregulation of EP4-Rs in L4 and L5 DRG did not result in a role for these receptors in chronic mechanoreflex sensitization. EP4-Rs are Gs-protein coupled receptors that stimulate adenyl cyclase to produce cAMP which initiates a downstream intracellular signaling cascade (54). Specifically, cAMP may sensitize piezo 2 channels through exchange protein directly activated by cAMP (16). Our finding that femoral artery ligation did not produce an increase in cAMP concentration may explain the fact that EP4-R blockade had no effect on the pressor response to dynamic hindlimb muscle stretch in ligated rats. A possible explanation for the lack of effect on cAMP concentration is an interaction produced by the femoral artery ligation-induced increase in DRG bradykinin 2 (B2) receptor expression reported by Lu et al. (33). Specifically, some B2 receptors are Gi-protein coupled receptors that inhibit adenyl cyclase and ultimately reduce the production of cAMP (28). Thus, the ligation-induced

upregulation of both EP4-Rs and B2 receptors may not result in a net increase in adenyl cyclase activity and cAMP concentration in DRG of ligated compared to freely perfused rats.

Our finding that TxA_2-R blockade reduced the pressor and cardioaccelerator response to dynamic stretch in ligated rats indicates that the COX-mediated chronic mechanoreflex sensitization present in this simulated PAD model (8) occurs, at least in part, through a TxA_2-R dependent mechanism. More specifically, the chronic sensitization appears to be driven by a femoral artery ligation-induced increase in DRG TxA_2-R protein expression and not the result of elevated basal levels COX-metabolites within skeletal muscles (8). Specifically, rat femoral artery ligation was found to have no effect on basal hindlimb skeletal muscle prostaglandin E2 (8) or TxB2 (the stable analog of TxA_2, 27) concentration. Our present finding is consistent with that of Leal et al. (27) where TxA_2-R blockade reduced the pressor response to static hindlimb muscle stretch in rats with ligated femoral arteries. As suggested above, however, the finding of Leal et al.(27) is somewhat difficult to interpret because static stretch of ligated hindlimb skeletal muscles does not consistently produce an exaggerated pressor response compared to that produced when freely perfused hindlimb skeletal muscles are statically stretched. Moreover, a static mechanical stimulus is quite different from a dynamic mechanical stimulus, likely in regards to both the classes of mechanically activated channels stimulated (52) and the cardiovascular consequences produced (61). The use of dynamic hindlimb muscle stretch in the present investigation was therefore an important extension of our recent work using the same mode of activation (8) and that of Leal et al. (27) in which static muscle stretch was used.

Blockade of TxA_2-R with daltroban likely reduced sensitization of mechanically activated channels on muscle afferent sensory endings of ligated rats in our experiments. If daltroban had reduced the pressor response to the non-mechanical stimulus of lactic acid injection, we would not have been able to rule out the possibility that TxA_2-R blockade resulted in a general/overall reduction in afferent excitability that made them less responsive to muscle stretch. Although outside the scope of the present investigation, the intracellular pathway connecting TxA_2-R and mechanically activated channels likely includes the fact that TxA_2-R are G_q-protein coupled receptors that, when stimulated, activate the phosphatidylinositol pathway resulting in formation of inositol trisphosphate (IP3) and diacylglycerol (18, 19). Rodent models of chronic pain have shown that an upregulation of IP3 signaling resulted in heightened mechanical sensitivity (63). Stimulation of IP3 receptors increases calcium release from the

endoplasmic reticulum and a rise in cytosolic calcium has been shown to potentiate mechanically activated piezo 2 mediated currents in vitro by decreasing activation threshold (16, 63).

Several experimental considerations warrant discussion. First, atherosclerosis develops slowly and results in a gradual, progressive narrowing of the arteries in PAD patients (45) whereas we instantaneously occluded a rat femoral artery ~72 hrs before experiments. Nevertheless, rat femoral artery ligation followed by ~72 hrs recovery replicates the blood flow patterns at rest and during exercise that are present in PAD patients in which limb blood flow is adequate to meet metabolic demand at rest but reserve blood flow capacity is significantly reduced during exercise (5, 35, 36). Second, the hindlimb muscle stretch model of isolated mechanoreflex activation stimulates mechanically activated channels by passively lengthening the muscle. Conversely, during skeletal muscle contractions mechanically activated channels are likely stimulated when skeletal muscles shorten and intramuscular pressure increases (17). Despite that difference, the vast majority of the thin fiber muscle afferents that are activated by stretch are also activated by muscle contraction, at least in the rat (57). Third, the hindlimb muscle stretch model used presently is specifically designed to isolate mechanical stimuli from contraction-induced metabolites. Consequently, our results are not able to shed light on the presence of, or possible mechanisms of, acute mechanoreflex sensitization that is likely to occur during skeletal muscle contractions. Finally, female rats were not used in this study and whether the present findings in male rats extend to female rats is an important future direction.

In summary, we investigated the role played by EP4-Rs and TxA_2-Rs, receptors associated with products of COX activity, in the chronic mechanoreflex sensitization present in the femoral artery ligated rat model of simulated PAD. We found that blockade of TxA_2-Rs, but not EP4-Rs, reduced the pressor and cardioaccelerator response to dynamic hindlimb skeletal muscle stretch, a model of mechanoreflex activation isolated from any contraction-induced metabolic stimulus. When considered in conjunction with our recent findings (8), the results indicate that chronic mechanoreflex sensitization in this simulated PAD model occurs, at least in part, through a TxA_2-R dependent mechanism that is mediated by increased DRG TxA_2-R protein expression.

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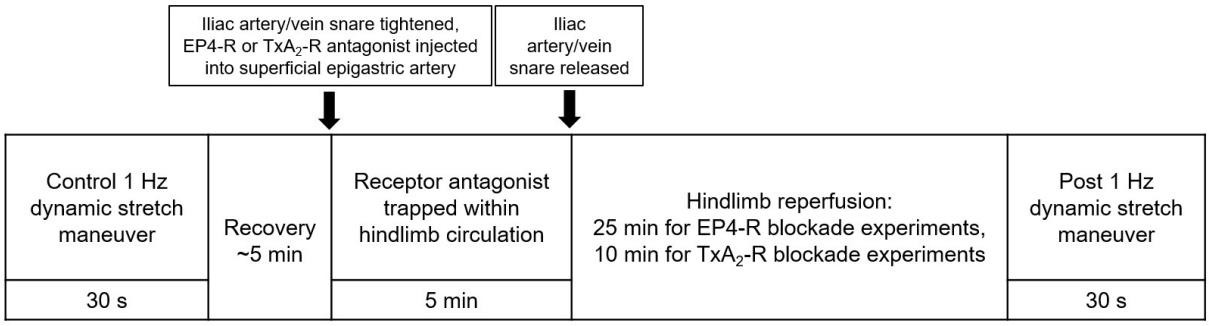
Table 2.1 ***Baseline mean arterial pressure (MAP) and heart rate (HR)***

Baseline MAP (mmHg)			
Experimental group	Control	Post condition	p-value
Freely perfused stretch (EP4-R antagonist, n=6)	103±11	97±10	0.45
Ligated stretch (EP4-R antagonist, n=8)	104±6	101±3	0.38
Freely perfused stretch (TxA_2-R antagonist, n=6)	111±10	110±8	0.83
Ligated stretch (TxA_2-R antagonist, n=11)	112±6	112±6	0.97
Ligated contraction (EP4-R antagonist, n=3)	101±6	104±2	0.74
Ligated stretch (1% DMSO, n=6)	97±5	101±6	0.38
Ligated stretch (TxA_2-R antagonist contralateral, n=4)	126±11	113±10	0.34
Ligated lactic acid injection (TxA_2-R antagonist, n=5)	130±11	132±7	0.82
Baseline HR (bpm)			
Experimental group	Control	Post condition	p-value
Freely perfused stretch (EP4-R antagonist, n=6)	489±10	490±14	0.93
Ligated stretch (EP4-R antagonist, n=8)	508±11	514±9	0.42
Freely perfused stretch (TxA_2-R antagonist, n=6)	510±20	514±13	0.68
Ligated stretch (TxA_2-R antagonist, n=11)	510±8	505±13	0.65
Ligated contraction (EP4-R antagonist i.a., n=3)	409±7	407±7	0.66
Ligated stretch (1% DMSO, n=6)	488±18	488±20	0.96
Ligated stretch (TxA_2-R antagonist contralateral, n=4)	510±23	515±25	0.34
Ligated lactic acid injection (TxA_2-R antagonist, n=5)	531±12	538±12	0.55

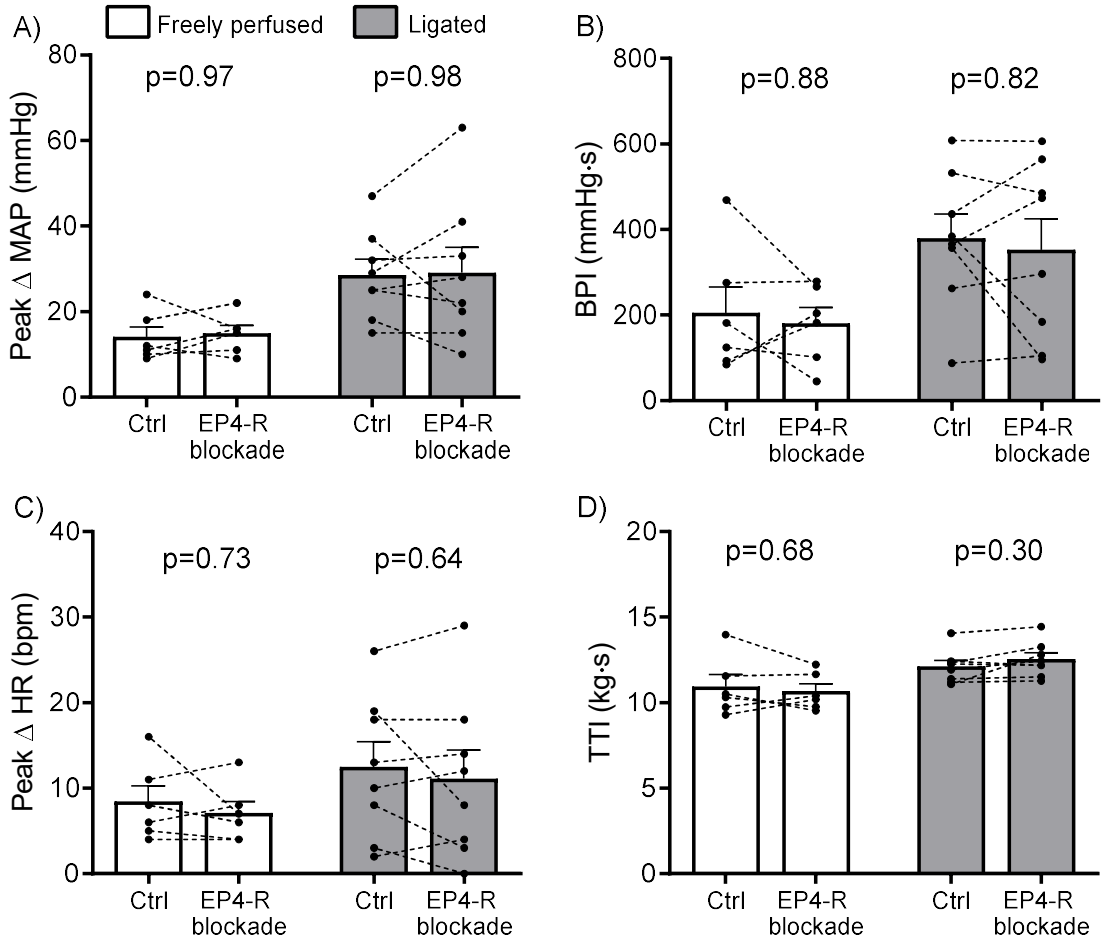
Data are expressed as mean±SEM and were compared with paired Student's t-tests. The experimental group is identified by whether the femoral artery was ligated or freely perfused and the primary experimental maneuver performed. The pharmacological agent injected into the arterial supply of the hindlimb and the sample size for each group is indicated in parentheses.

Figure 2.1. ***Primary mechanoreflex experimental protocol***



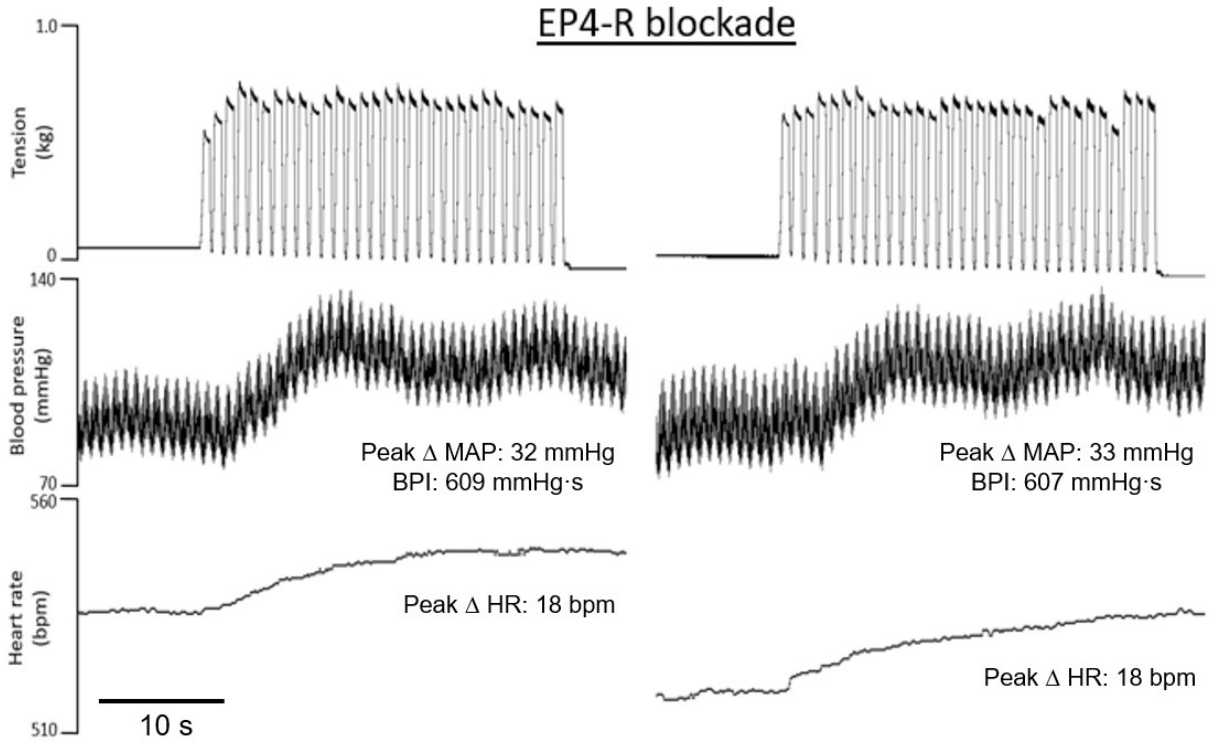
Schematic representation of the primary mechanoreflex experimental protocol.

Figure 2.2. ***Effect of endoperoxide 4 (EP4) receptor blockade with*** **L161982** ***on isolated mechanoreflex activation***



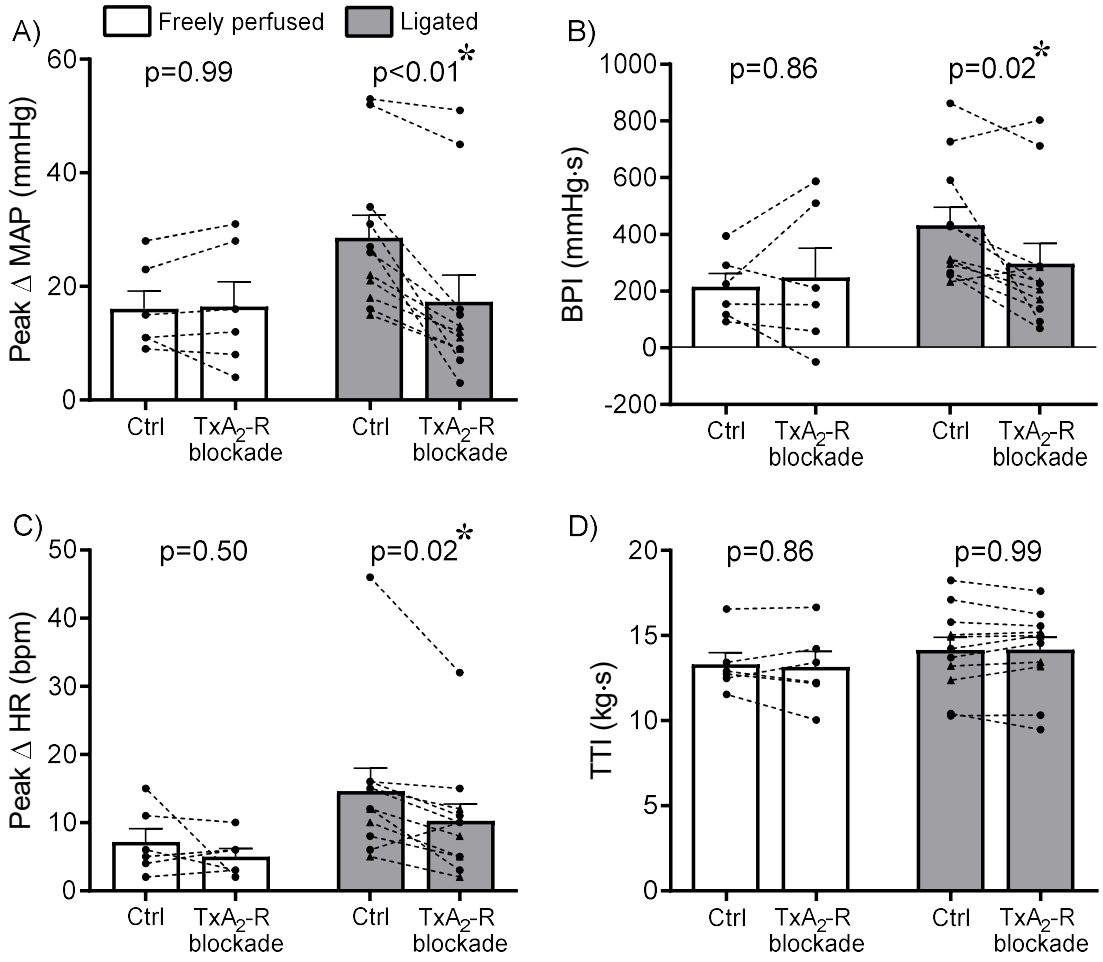
The peak Δ mean arterial pressure (peak Δ MAP, *A*), blood pressure index (BPI, *B*), and peak Δ heart rate (peak Δ HR, *C*) response to 30 seconds of 1 Hz dynamic stretch before (Ctrl) and after injection of the EP4-R antagonist L161982 (1 μg) into the arterial supply of the hindlimb. The tension-time index (Δ TTI) was not different between conditions (*D*) for either freely perfused (n=6) or ligated (n=8) rats. Data were analyzed with Sidak multiple comparisons tests and are expressed as mean±SEM overlaid with individual responses.

Figure 2.3. ***Original data tracing before and after EP4 receptor blockade during isolated mechanoreflex activation***



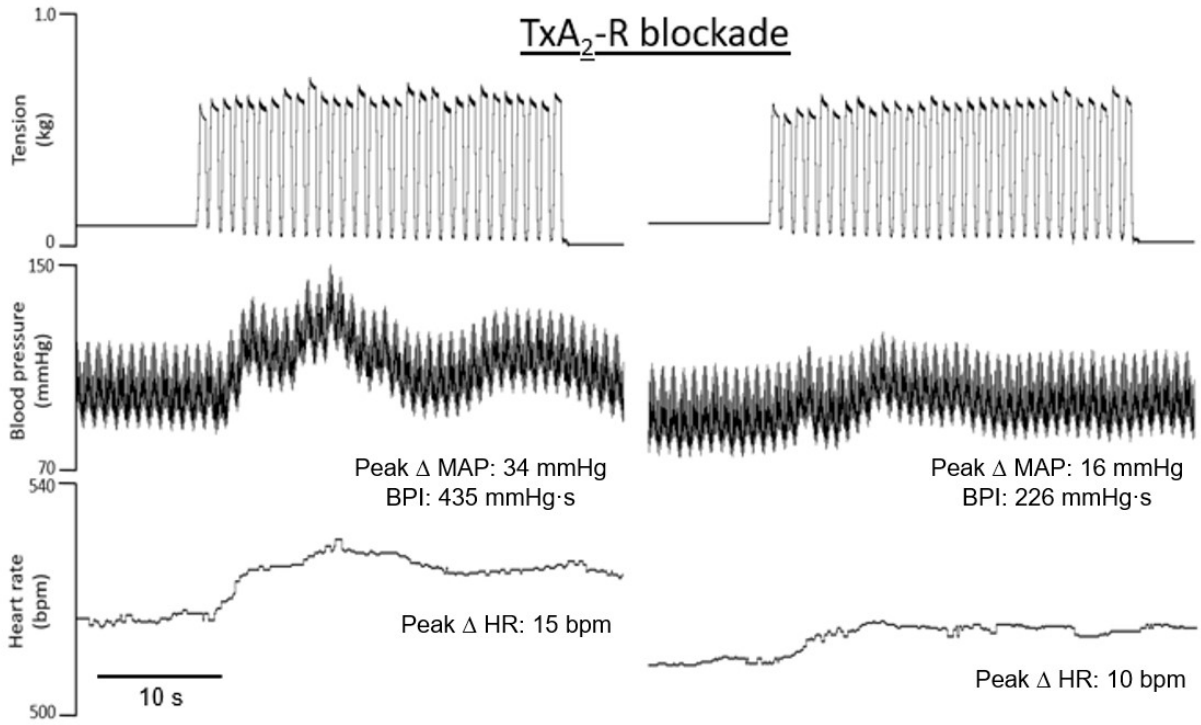
Examples of original tracings of the blood pressure and heart rate response to dynamic stretch before (*left*) and after (*right*) injection of the EP4-R antagonist L161982 into the arterial supply of the hindlimb of a ligated rat. MAP = mean arterial pressure. BPI = blood pressure index. HR = heart rate.

Figure 2.4. ***Effect of thromboxane A_2 (TxA_2) receptor blockade with daltroban on isolated mechanoreflex activation***



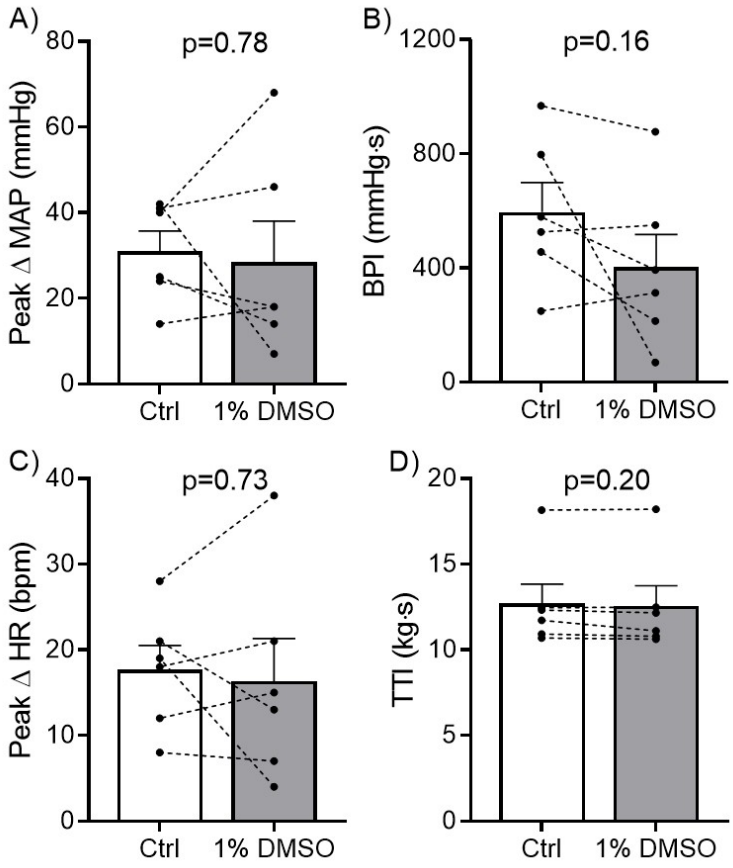
The peak Δ mean arterial pressure (peak Δ MAP, *A*), blood pressure index (BPI, *B*), and peak Δ heart rate (peak Δ HR, *C*) response to 30 seconds of 1 Hz dynamic stretch before (Ctrl) and after injection of the TxA_2-R antagonist daltroban (80 µg) into the arterial supply of the hindlimb. The tension-time index (Δ TTI) was not different between conditions (*D*) for either freely perfused (n=6) or ligated (n=11) rats. Data were analyzed with Sidak multiple comparisons tests and are expressed as mean±SEM overlaid with individual responses. Circles represent daltroban dissolved in 1% DMSO and triangles represent daltroban dissolved in 100 mM sodium carbonate. Asterisks (*) indicate values were significantly different between conditions ($p<0.05$).

Figure 2.5. ***Original data tracing before and after TxA_2 receptor blockade during isolated mechanoreflex activation***



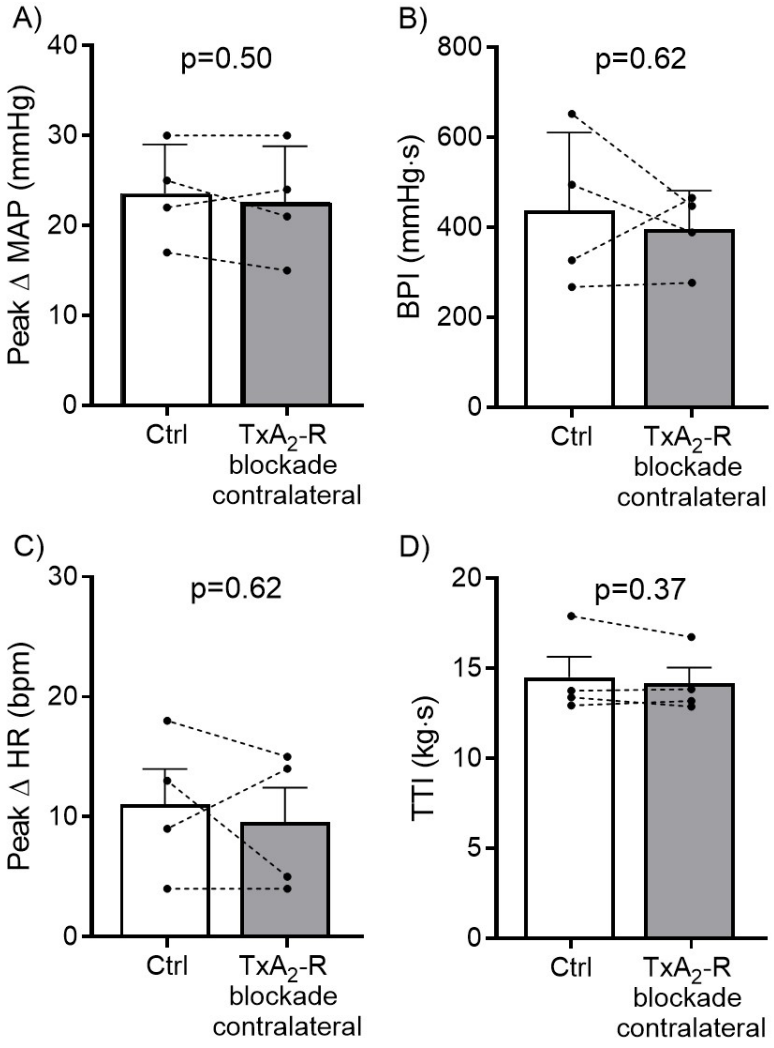
Examples of original tracings of the blood pressure and heart rate response to dynamic stretch before (*left*) and after (*right*) injection of the TxA_2-R antagonist daltroban into the arterial supply of the hindlimb of a ligated rat. MAP = mean arterial pressure. BPI = blood pressure index. HR = heart rate.

Figure 2.6. ***Effect of 1% DMSO on isolated mechanoreflex activation***



The peak Δ mean arterial pressure (peak Δ MAP, *A*), blood pressure index (BPI, *B*), and peak Δ heart rate (peak Δ HR, *C*) response to 30 seconds of 1 Hz dynamic stretch before (Ctrl) and after injection of 1% DMSO in to the arterial supply of the hindlimb of ligated rats (n=6). The tension-time index (Δ TTI) was not different between conditions (*D*). Data were analyzed with a paired Student's t-test and are expressed as mean±SEM overlaid with individual responses.

Figure 2.7. ***Effect of systemic injection of daltroban on isolated mechanoreflex activation***



The peak Δ mean arterial pressure (peak Δ MAP, *A*), blood pressure index (BPI, *B*), and peak Δ heart rate (peak Δ HR, *C*) response to 30 seconds of 1 Hz dynamic stretch before (Ctrl) and after injection of the TxA_2-R antagonist daltroban into the arterial supply of the contra-lateral hindlimb of ligated rats (n=4). The tension-time index (Δ TTI) was not different between conditions (*D*). Data are analyzed with paired Student's t-tests and expressed as mean±SEM overlaid with individual responses.

Chapter 3 - Sensory Neuron Inositol-1,4,5-Trisphosphate (IP_3) Receptors Contribute to Chronic Mechanoreflex Sensitization in Rats with Simulated Peripheral Artery Disease

Abstract

The mechanoreflex is exaggerated in patients with peripheral artery disease (PAD) and in a rat model of simulated PAD in which a femoral artery is chronically (~72hrs) ligated. We found recently that, in rats with a ligated femoral artery, blockade of thromboxane A2 (TxA_2) receptors on the sensory endings of thin fiber muscle afferents reduced the pressor response to 1 Hz repetitive/dynamic hindlimb skeletal muscle stretch (a model of mechanoreflex activation isolated from contraction-induced metabolite production). Conversely, we found no effect of TxA_2 receptor blockade in rats with freely perfused femoral arteries. Here we extended the isolated mechanoreflex findings in "ligated" rats to experiments evoking dynamic hindlimb skeletal muscle contractions. We also investigated the role played by inositol 1-4-5-trisphosphate (IP_3) receptors, receptors associated with intracellular signaling linked to TxA_2 receptors, in the exaggerated response to dynamic mechanoreflex and exercise pressor reflex activation in ligated rats. Injection of the TxA_2 receptor antagonist daltroban into the arterial supply of the hindlimb reduced the pressor response to 1 Hz dynamic contraction in ligated but not "freely perfused" rats. Moreover, injection of the IP_3 receptor antagonist xestospongin C into the arterial supply of the hindlimb reduced the pressor response to 1 Hz dynamic stretch and contraction in ligated but not freely perfused rats. These findings demonstrate that, in rats with a ligated femoral artery, sensory neuron TxA_2 receptor and IP_3 receptor mediated signaling contributes to a chronic sensitization of the mechanically activated channels associated with the mechanoreflex and the exercise pressor reflex.

Introduction

Skeletal muscle contraction during exercise stimulates mechanically activated (MA) channels located on the sensory endings of group III and IV thin fiber skeletal muscle afferents (35-37, 48, 49). The stimulation of these channels activates the mechanoreflex which, along with the metaboreflex, is an important constituent of the exercise pressor reflex (34, 53, 74). The exercise pressor reflex plays an integral role in increased sympathetic nervous system activity, heart rate, and blood pressure during exercise (1, 2). The mechanoreflex is crucial for normal blood pressure regulation during exercise in health but becomes augmented during exercise in many forms of chronic disease which contributes to aberrant sympathoexcitation and augmented blood pressure reactivity (27, 41, 50, 51, 54-56, 59, 71). Of particular relevance for this investigation, the mechanoreflex was suggested to contribute to the exaggerated pressor response to rhythmic lower leg exercise in patients with peripheral artery disease (PAD) compared to that found in aged-matched healthy control counterparts (21, 55, 56). Moreover, the mechanoreflex was found to be exaggerated during 1 Hz rhythmic hindlimb muscle contractions in a rat model of simulated PAD in which a femoral artery was ligated ~72 hours before experimentation compared to the mechanoreflex found in rats in which the femoral artery was patent (15). Acute exaggerations in blood pressure reactivity during exercise are an independent risk factor for cardiovascular morbidity and mortality in both healthy and clinical populations (18, 78). Thus, identifying the mechanisms underlying the exaggerated mechanoreflex in PAD carries clinical importance.

Our laboratory has used a 1 Hz repetitive/dynamic rat hindlimb skeletal muscle stretch protocol that is based on original work by Stebbins and colleagues (17, 72) as an experimental model to investigate MA channel stimulation and mechanoreflex activation isolated from muscle contraction-induced metabolite influences (10, 38, 61, 65). Using this model, we found that the pressor and renal sympathetic nerve activity (RSNA) response to 30 seconds of dynamic muscle stretch was larger when evoked from hindlimb muscles associated with a chronically ligated femoral artery compared to responses evoked from the contralateral hindlimb muscles which were freely perfused (38). Moreover, rat femoral artery ligation was found recently to have no effect on L4 and L5 dorsal root ganglia (DRG) protein expression of piezo channels (15), a novel class of MA channel that has been suggested to underlie mechanoreflex activation (14, 15, 27, 28, 65). Together, these findings suggest that the exaggerated mechanoreflex in rats with a

ligated femoral artery is due, at least in part, to a chronic sensitization of MA channels located on the sensory endings of thin fiber muscle afferents. That is consistent with a large body of literature demonstrating that G protein linked second messenger signaling cascades within sensory neurons may potentiate MA channel function; particularly in chronic inflammatory conditions (22, 24, 60, 67). In further investigation of the mechanisms of chronic mechanoreflex sensitization, we found recently that inhibition of the cyclooxygenase (COX) enzyme (10) and blockade of COX metabolite associated thromboxane A_2 (TxA_2) receptors (61) reduced the pressor response to dynamic hindlimb skeletal muscle stretch in rats with a ligated femoral artery. Conversely, we found no effect of either intervention on the pressor response to muscle stretch in rats with freely perfused hindlimbs (10, 61). These findings were supported by molecular evidence demonstrating that rat femoral artery ligation increased TxA_2 receptor protein expression in L4 and L5 DRG tissue (9). Thus, there is experimental support for the conclusion that, in rats with a ligated femoral artery, COX metabolite/TxA_2 receptor signaling increases the responsiveness of MA channels on thin fiber muscle afferents that contribute to mechanoreflex activation.

The specific intracellular signaling mechanisms within sensory neurons that mediate TxA_2 receptor-induced mechanoreflex sensitization have not been investigated. In sensory neurons, TxA_2 receptors are coupled to G_q proteins that, when stimulated, activate phospholipase C (PLC) to induce increases in diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) formation (31, 33, 58). Although both of these ubiquitous intracellular signaling molecules have been linked to sensory neuron sensitization to various stimuli (7), a possible for role IP_3 in mechanoreflex sensitization holds indirect experimental support. IP_3 binds to its intracellular receptor on the endoplasmic reticulum which results in the release of stored calcium into the cytosol. Elevated cytosolic calcium concentration has been shown to sensitize MA piezo channel-mediated currents in HEK293 cells (24). Moreover, sensory neuron IP_3 receptors have been found to play an important role in the development of mechanical allodynia in mice (82). Together, these findings raise the possibility that amplified IP_3 receptor signaling may contribute to a chronic sensitization of MA channels and the mechanoreflex in rats with simulated PAD induced by chronic femoral artery ligation.

Based on the information above, the present investigation was undertaken with two primary goals. First, we sought to confirm that the role played by TxA_2 receptors in the pressor

response to dynamic hindlimb muscle stretch in rats with a ligated femoral artery reported recently by our laboratory (62) reflected a role for TxA_2 receptors in the exaggerated exercise pressor reflex in this simulated PAD model. Second, we investigated the role played by sensory neuron IP_3 receptors in the chronic sensitization of MA channels and the exaggerated mechanoreflex and exercise pressor reflex in rats with a ligated femoral artery. We tested the hypotheses that, in decerebrate, unanesthetized rats, 1) injection of the TxA_2 receptor antagonist daltroban (80 μg) into the arterial supply of the hindlimb reduces the pressor and cardioaccelerator response to 1 Hz dynamic hindlimb skeletal muscle contraction to a greater extent in rats with a ligated femoral artery than in sham-operated rats with freely perfused hindlimb muscles, and 2) injection of the IP_3 receptor antagonist xestospongin C (XeC, 5 μg) into the arterial supply of the hindlimb reduces the pressor and cardioaccelerator response to 1 Hz dynamic hindlimb skeletal muscle stretch and contraction in rats with a ligated femoral artery but not in sham-operated rats with freely perfused hindlimb muscles.

Methods and Materials

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 (~12-15 weeks old) male Sprague-Dawley rats (n=78, average body weight: 410±5 g; Charles River Laboratories). The rats were housed two per cage in temperature (maintained at 22°C) and light (12-12 hr light-dark cycle)-controlled accredited facilities with standard rat chow and water provided ad libitum. At the end of each experiment, the decerebrated rats (see below) were killed by an intravenous injection of saturated (>3 mg/kg) potassium chloride.

Femoral artery ligation/sham procedure. Of the 78 rats in this investigation, 63 had their left femoral artery ligated ~72 hrs before the terminal experimental protocol was performed. Briefly, rats were anesthetized with 2% isoflurane anesthesia (balance O_2) and their left femoral artery was surgically exposed and ligated tightly with 5-0 silk suture ~3–5 mm distal to the inguinal ligament. In 15 rats, a sham ligation procedure was performed in which the left femoral artery was surgically exposed, and 5-0 suture was passed under the femoral artery but not tied. Thus, the hindlimb remained freely perfused following the sham procedure. In both "ligated" and "freely perfused" rats (referred to as such from here forward for simplicity), the incisions were closed, meloxicam was administered (1-2 mg/kg s.c.) as an analgesic, and the rats were monitored daily until the final experiment.

Surgical procedures for experimental protocols. On the day of the experiment, rats were anesthetized with ~2% isoflurane (balance O_2). Adequate depth of anesthesia was confirmed by the absence of toe-pinch and blink reflexes. The trachea was cannulated, and the lungs were mechanically ventilated (Harvard Apparatus model 683) with the gaseous anesthetic until the decerebration was completed (see below). In all rats, the right jugular vein and both common carotid arteries were cannulated with PE-50 catheters for the administration of fluids/drugs, measurement of arterial blood pressure (Physiological Pressure Transducer, AD Instruments), and sampling of arterial blood gases (ABL80 Flex, Radiometer). Heart rate (HR) was measured by electrocardiogram. The left calcaneus bone was severed and the triceps surae (gastrocnemius, soleus and plantaris) muscles were exposed by reflecting the overlying skin and skeletal muscles. A string was then tied to the distal Achilles tendon and severed calcaneus which linked the

triceps surae muscles to a force transducer (Grass FT03) and rack and pinion that could be turned manually. In the 15 sham rats and 58 of the ligated rats, the left superficial epigastric artery was cannulated with a PE-8 catheter whose tip was located near the junction of the superficial epigastric artery and the femoral artery. In those rats in which a catheter was placed in the left superficial epigastric artery, a reversible snare was placed around the left iliac artery and vein (i.e., proximal to the location of the catheter placed in the superficial epigastric artery). For rats in which dynamic hindlimb skeletal muscle contraction was performed (n=29), the left sciatic nerve was exposed.

After the initial surgical procedures, all rats were placed in a Kopf stereotaxic frame with clamps placed around the pelvis. Dexamethasone (0.2 mg i.v.) was injected to minimize brainstem edema. A precollicular decerebration was performed and all neural tissue rostral to the superior colliculus was aspirated. After the decerebration was completed, anesthesia was terminated and the lungs were mechanically ventilated with room air. The decerebration procedure was performed because anesthesia has been shown to depress the exercise pressor reflex in the rat (68). Arterial blood gases and pH were measured periodically with a blood gas analyzer and maintained within normal limits ($PaCO_2$: 35–45 mmHg, PaO_2: ~100 mmHg, pH: 7.35–7.45) by adjusting ventilation and/or administering intravenous sodium bicarbonate (8.5%). Core temperature was measured by a rectal probe and maintained at ~37-38°C by an automated heating system (Harvard Apparatus) and heat lamp. For all rats in which dynamic hindlimb muscle stretch was performed (49 of the 78 rats) the paralytic pancuronium bromide (1 mg/kg i.v.) was injected prior to the initiation of any stretch maneuver in order to prevent any spontaneous or reflex muscle contraction which would produce a metabolic stimulus.

Exercise pressor reflex activation protocol. The control dynamic hindlimb muscle contraction maneuver was performed at least 60 minutes following termination of isoflurane anesthesia. To begin, baseline muscle tension was set to ~100 g and baseline blood pressure and HR were measured for ~30 seconds. The sciatic nerve was then electrically stimulated using stainless steel electrodes for 30 seconds at a voltage of ~1.5x motor threshold (0.01 ms pulse duration, 500 ms train duration, 40 Hz frequency) which produced 1 Hz repetitive/dynamic contractions of the triceps surae muscles. ~10-15 minutes following the control contraction maneuver, the snare on the left iliac artery and vein was tightened and the appropriate solution (see below) was injected into the arterial supply of the hindlimb via the left superficial epigastric

artery catheter. After the appropriate time had passed, the left iliac artery and vein snare was released and the hindlimb was allowed to reperfuse before the muscle contraction protocol was repeated as described above. At the end of the experiment, to ensure that the increase in blood pressure and HR during contraction were not due to electrical activation of the axons of the thin fiber afferents in the sciatic nerve, we administered the paralytic pancuronium bromide (1 mg/kg i.v.) and the sciatic nerve was stimulated for 30 s with the same parameters as those used to elicit contraction. No increase in blood pressure or HR was observed during the stimulation period after the administration of pancuronium bromide in any of the experiments for which data are reported which indicates the increases in blood pressure during contractions were reflex in nature. Additionally, at the end of each experiment Evans blue dye was injected in the same manner as the experimental solution to confirm that the injectate had access to the triceps surae muscle circulation.

The following muscle contraction protocols were performed. The injectate, the time the iliac artery and vein snare was pulled tight, the time the hindlimb was allowed to reperfuse, and the sample sizes are indicated.

1) Daltroban (TxA_2 receptor antagonist, 80 µg dissolved in 0.4 ml of 1% DMSO), snare tight for 5 minutes, hindlimb reperfused for 10 minutes, n=6 freely perfused rats and n=10 ligated rats.

2) 0.4 ml of 1% DMSO (vehicle for daltroban), snare tight for 5 minutes, hindlimb reperfused for 10 minutes, n=5 ligated rats.

3) XeC (IP_3 receptor antagonist, 5 µg dissolved in 0.2 ml of 0.02% ethanol), snare tight for 10 minutes, hindlimb reperfused for 30 minutes, n=4 freely perfused and n=5 ligated rats.

Isolated mechanoreflex activation protocol. The control dynamic hindlimb muscle stretch maneuver was performed at least 60 minutes following termination of isoflurane anesthesia. To begin, baseline muscle tension was set at ~100 g and baseline MAP and HR were collected for 30 seconds. An experienced investigator then elicited repetitive/dynamic triceps suare muscle stretch for 30 seconds by manually turning the rack and pinion at a 1 Hz frequency with the aid of a metronome. The investigator aimed to develop ~0.6 to 0.8 kg of tension during each dynamic stretch maneuver because that is the tension typically developed during hindlimb muscle contractions in decerebrate rat preparations (14, 15, 38). Moreover, the investigator aimed for consistent levels of tension development for each individual dynamic stretch although

slight variability in tension development was often present. The dynamic stretch protocol was adapted from that described by Daniels et. al. (17). ~5 minutes following the control stretch maneuver, the snare on the left iliac artery and vein was tightened and the appropriate solution (see below) was injected into the arterial supply of the hindlimb via the left superficial epigastric artery catheter. After the appropriate time had passed, the left iliac artery and vein snare was released and the hindlimb was allowed to reperfuse before the dynamic stretch protocol was repeated as described above. At the end of each experiment, Evans blue dye was injected in the same manner as the experimental solution to confirm that the injectate had access to the triceps surae muscle circulation.

The following isolated mechanoreflex protocols were performed. The injectate, the time the iliac artery and vein snare was pulled tight, the time the hindlimb was allowed to reperfuse, and the sample sizes are indicated.

1) XeC (IP_3 receptor antagonist, 5 µg dissolved in 0.2 ml of 0.02% ethanol), snare tight for 10 minutes, hindlimb reperfused for 30 minutes, n=9 freely perfused rats and n=14 ligated rats.

2) 0.2 ml of 0.02% ethanol (vehicle for XeC), snare tight for 10 minutes, hindlimb reperfused for 30 minutes, n=6 ligated rats.

3) 2-Aminoethoxydiphenylborane (2-APB, IP_3 receptor antagonist, 170 µg dissolved in 0.2 ml of 3.75% DMSO), snare tight for 10 minutes, hindlimb reperfused for 30 minutes, n=6 ligated rats.

4) 0.2 ml of 3.75% DMSO (vehicle for 2-APB), snared for 10 minutes, hindlimb reperfused for 30 minutes, n=4 ligated rats.

5) Capsaicin (TRPV1 agonist, 0.5 µg in 0.25 ml), snared for 10 minutes, hindlimb reperfused for 30 minutes, n=6 ligated rats.

In an additional group of five ligated rats, dynamic hindlimb skeletal muscle stretch maneuvers were performed before and after XeC (5 µg) was injected into the right jugular vein and therefore allowed to circulate systemically. Forty minutes elapsed between the i.v. injection of XeC and the subsequent stretch maneuver exactly as described above in protocol 1 when XeC was injected into the arterial supply of the hindlimb.

Lactic acid injection protocol. In another group of four ligated rats, an injection of 0.2 ml of 24 mM lactic acid into the arterial supply of the hindlimb was performed before and after XeC

(5 μg) was injected into the arterial supply of the hindlimb exactly as described above in protocol 1. Forty minutes elapsed between the injection of the XeC and the subsequent lactic acid injection maneuver also as described above in protocol 1.

Drugs. Daltroban was dissolved in 100% DMSO and diluted to a final concentration of 80 μg in 0.4 ml of 1% DMSO. Dose and timing were based on previous use of the drug by our laboratory (11, 61) and Leal et al. (40). Doses of XeC and 2-APB were calculated from in vitro molarity concentrations assuming a hindlimb blood volume of ~15 ml (79). XeC was dissolved initially in 10% ethanol and diluted to a final concentration of 5 μg in 0.25 ml of 0.02% ethanol. The dose and timing of the XeC protocol were based on in vitro evidence that ~700 nM (12, 25) inhibited IP_3 receptors. 2-APB was dissolved in 100% DMSO and diluted to a final concentration of 170 μg in 0.2 ml of 3.75% DMSO. The dose of 2-APB was based on in vitro evidence that 50 μM inhibited IP_3 receptors (45). Capsaicin was dissolved in pure ethanol and Tween 20 then diluted in saline to yield a final concentration of 2 μg/ml. The dose was based on previous use of capsaicin to stimulate TRP channels (23, 70).

Data analysis. Data were collected with a PowerLab and LabChart data acquisition system (AD Instruments). Arterial blood pressure, electrocardiogram, and muscle tension were measured, mean arterial pressure (MAP) and HR were calculated, and all data were displayed in real time and recorded for offline analysis. Baseline MAP and HR were determined from the 30 s baseline periods that preceded each maneuver. The peak pressor (peak Δ MAP) and cardioaccelerator (peak Δ HR) responses were calculated as the difference between the peak values wherever they occurred during the 30 second maneuvers and their corresponding baseline value. The tension-time indexes (TTIs) and blood pressure indexes (BPIs) were calculated by integration of the area under signal during the stretch or contraction maneuver and subtracting the integrated area under the signal during the corresponding baseline period. The time course of the increase in MAP and HR were plotted as Δ MAP and Δ HR from baseline over the course of the 30 second contraction or stretch maneuvers. Data are expressed as mean±SEM and were analyzed with paired Student's t-tests, two-way repeated measures ANOVAs and/or Sidak multiple comparisons tests as appropriate. Statistical significance was accepted at $p \leq 0.05$.

Results

Effect of the TxA_2 receptor blockade with daltroban on the exercise pressor reflex. In freely perfused rats (n=6), injection of the TxA_2 receptor antagonist daltroban into the arterial supply of the hindlimb had no effect on the pressor or cardioaccelerator response to dynamic hindlimb skeletal muscle contraction (Fig. 1 and 2). In contrast, in ligated rats (n=10) daltroban significantly reduced the pressor and cardioaccelerator response to contraction (Fig. 1 and 2). The Δ TTI of the contraction maneuver was not different between control and daltroban conditions in either group (Fig. 1D). Baseline MAP and HR were not different between conditions in either group (Table 1).

In control experiments in ligated rats (n=5), 0.4 ml of 1% DMSO in saline (the vehicle for daltroban) had no effect on the peak Δ MAP (control: 22±4, 1% DMSO: 20±2 mmHg, p=0.51), BPI (control: 410±95, 1% DMSO: 386±57 mmHg·s, p=0.80), or peak Δ HR (control: 27±5, 1% DMSO: 33±6 bpm, p=0.07) response to dynamic contraction. The Δ TTI of the dynamic contraction maneuver was not different between control (9±1 kg·s) and 1% DMSO (8±1) kg·s, p=0.24) conditions. Baseline MAP and HR were not different between conditions (Table 1).

Effect of IP_3 receptor blockade with XeC on isolated mechanoreflex and exercise pressor reflex activation. In sham rats (n=9), injection of the IP_3 receptor antagonist XeC into the arterial supply of the hindlimb did not reduce, and actually slightly increased at several time points, the pressor or cardioaccelerator response to dynamic stretch (Fig. 3 and 4). In contrast, in ligated rats (n=14) XeC significantly reduced the pressor and cardioaccelerator response to stretch (Fig. 3 and 4). The Δ TTI of the stretch maneuver was not different between control and XeC conditions in either group (Fig. 4D). Baseline MAP and HR were not different between conditions (Table 1). An example of an original tracing of the effect of XeC during dynamic stretch in a rat in which the femoral artery was previously ligated shown in the top panels of Fig 5.

In vehicle control experiments in ligated rats (n=6), 0.02% ethanol (the vehicle for XeC) had no effect on the peak Δ MAP (control: 35±7, 0.02% ethanol: 32±5 mmHg, p=0.49), BPI (control: 568±147, 0.02% ethanol: 534±110 mmHg·s, p=0.77), or peak Δ HR (control: 15±4, 0.02% ethanol: 12±3 bpm, p=0.37) response to stretch. The Δ TTI of the stretch maneuver was not different between control (13±1 kg·s) and 0.02% ethanol (13±1 kg·s, p=0.26) conditions. Baseline MAP and HR were not different between conditions (Table 1).

In systemic control experiments in ligated rats (n=5), injection of XeC into the jugular vein to allow it to circulate systemically had no effect on the peak Δ MAP (control: 47±7, i.v. XeC: 43±8 mmHg, p=0.49), BPI (control: 585±86, i.v. XeC: 549±70 mmHg·s, p=0.70), or peak Δ HR (control: 8±1, i.v. XeC: 9±1 bpm, p=0.52) response to stretch. The Δ TTI of the dynamic stretch maneuver was not different between control (12±1 kg·s) and i.v. XeC (12±1 kg·s, p=0.37) conditions. The Δ TTI of the stretch maneuver was not different between control and i.v. XeC conditions. Baseline MAP and HR were not different between conditions (Table 1). These results suggest that the effect of XeC when it was injected into the arterial supply of the hindlimb of ligated rats on the pressor response to stretch is attributable to effects on the sensory endings of thin fiber muscle afferents and not effects elsewhere in the mechanoreflex arc such as the brainstem and/or the spinal cord.

In "off target" control experiments in ligated rats (n=4), injection of XeC into the arterial supply of the hindlimb had no effect on the peak Δ MAP (control: 35±5, XeC: 41±9 mmHg, p=0.46) or peak Δ HR (control: 4±2, XeC: 7±2 bpm, p=0.07) produced in response to the injection of lactic acid into the arterial supply of the hindlimb. Baseline MAP and HR were not different between conditions (Table 1). These results suggest that the effect of XeC when it was injected into the arterial supply of the hindlimb on the pressor response to stretch is most likely attributable to an interruption of the cellular signaling between IP_3 receptors and MA channels and is not attributable to a local "off-target" effect such as the inhibition of voltage-gated sodium channels.

In experiments extending our isolated mechanoreflex findings to experiments evoking the exercise pressor reflex, injection of XeC into the arterial supply of the hindlimb of freely perfused rats (n=4) did not reduce the pressor or cardioaccelerator response to dynamic contraction (Fig. 6 and 7). In contrast, in ligated rats (n=5) XeC significantly reduced the pressor and cardioaccelerator response to contraction (Fig. 6 and 7). The Δ TTI of the contraction maneuver was not different between control and XeC conditions (Fig. 6D). Baseline MAP and HR were not different between conditions (Table 1). An example of an original tracing of the effect of XeC during dynamic contraction is shown in the bottom panels of Fig. 5.

Effect of 2-APB on isolated mechanoreflex activation. To provide further evidence for a role of IP_3 receptors in the exaggerated mechanoreflex in ligated rats, we investigated the effect of injection of a different IP_3 receptor antagonist, 2-APB, into the arterial supply of the hindlimb

on the pressor response to stretch. In 6 ligated rats, we found that 2-APB significantly reduced the pressor and cardioaccelerator response to stretch (Fig. 8). The Δ TTI of the stretch maneuver was not different between control and 2-APB conditions. Baseline MAP and HR were not different between conditions (Table 1).

In control experiments in ligated rats (n=4), injection of 0.2 ml of 3.75% DMSO (the vehicle for 2-APB) into the arterial supply of the hindlimb had no effect on the peak Δ MAP (control: 34±7, 3.75% DMSO: 30±5 mmHg, p=0.18), BPI (control: 555±216, 3.75% DMSO: 583±128 mmHg·s, p=0.79), or peak Δ HR (control: 13±5, 3.75% DMSO: 10±3 bpm, p=0.28) response to stretch. The Δ TTI of the stretch maneuver was not different between control (16±1 kg·s) and 3.75% DMSO (16±1 kg·s, p=0.85) conditions. Baseline HR, but not MAP, was significantly higher in the vehicle condition compared to control (Table 1).

In addition to blocking IP_3 receptors, 2-APB has also been shown to stimulate TRP channels including transient receptor potential cation channel subfamily V member 1 (TRPV1) (30, 70). The possibility that 2-ABP stimulated TRPV1 channels when it was injected into the arterial supply of the hindlimb in our experiments was raised by the fact that injection of 2-ABP resulted in pressor response (peak Δ MAP) of 60±7 mmHg. Therefore, we investigated the effect of TRPV1 stimulation with capsaicin on the pressor response to stretch. Injection of capsaicin into the arterial supply of the hindlimb produced a peak pressor response of 52±5 mmHg in 6 ligated rats. More importantly, capsaicin significantly reduced the pressor and cardioaccelerator response to stretch (Fig. 9). The Δ TTI of the stretch maneuver was not different between control and capsaicin conditions. Baseline MAP and HR were significantly higher in the post-capsaicin condition compared to control (Table 1).

Discussion

We investigated the role of sensory neuron TxA_2 receptors and IP_3 receptors in the exaggerated mechanoreflex and exercise pressor reflex in a rat model of simulated PAD in which a femoral artery was ligated for 72 hours. We first extended our recent finding that TxA_2 receptors contribute to chronic mechanoreflex sensitization in ligated rats (61) by confirming a role for TxA_2 receptors in the exaggerated exercise pressor reflex in ligated rats. The present investigation also provides the first evidence that IP_3 receptor signaling, a component of second messenger signaling associated with Gq protein coupled receptors such as TxA_2 receptors, contributes to the chronic sensitization of the MA channels that underlie dynamic mechanoreflex and exercise pressor reflex activation.

The 1 Hz hindlimb skeletal muscle contraction maneuver used herein replicates the rhythmic nature of muscle contractions present during locomotor movement. Although undoubtedly a mixed mechanical and metabolic stimulus, the maneuver elicits a particularly robust mechanical stimulus as evidenced by its production of reflex RSNA bursts in sync with skeletal muscle tension development (14, 38). Thus, we believe our finding that TxA_2 receptor blockade with daltroban reduced the cardiovascular responses to dynamic muscle contraction in ligated rats reflects a contribution to the sensitization of the MA channels that underlie mechanoreflex activation (61). Specifically, the effect of TxA_2 receptor blockade on the exercise pressor reflex in ligated rats likely reflects, at least in part, the chronic sensitization of MA channels that exists even in the absence of contraction-induced metabolite production (61). Acute MA channel sensitization mediated by possible contraction-induced elevations in COX metabolites (29, 46) may have occurred additively with the chronic sensitization to produce the overall TxA_2 receptor-mediated mechanoreflex contribution to the exercise pressor reflex in our experiments. A role for TxA_2 receptors in the metaboreflex component of the exercise pressor reflex in ligated rats is also possible (40). We did not find a role for TxA_2 receptors in the exercise pressor reflex in freely perfused rats in this investigation, although the possibility of redundancy among the receptors evoking the exercise pressor reflex in health must be considered (73).

XeC is a potent, selective, cell-membrane permeable molecule isolated from a marine sponge species (xestospongia) (25). In vitro evidence from multiple cell types has demonstrated that XeC inhibited IP_3 receptors and prevented the release of internal calcium stores into the

cytosol (3, 25, 57, 64, 75). XeC may work through a non-competitive mechanism in which the calcium channel pore is blocked and/or an allosteric mechanism that uncouples IP_3 binding from calcium release (25). The dose of XeC used presently was calculated based on in vitro evidence that ~700 nM blocks IP_3-induced calcium release from endoplasmic reticulum stores (25). Specifically, 5 ug of XeC distributed throughout an estimated rat hindlimb volume of 15 ml (79) results in an estimated concentration of ~746 nM. Our finding that XeC did not reduce the pressor or cardioaccelerator response to dynamic hindlimb muscle stretch or contraction in freely perfused rats is consistent with our findings that TxA_2 receptor blockade did not reduce the pressor response to hindlimb muscle stretch (62) or contraction (present data) in freely perfused rats. Moreover, we reported recently that COX inhibition (10, 63) and blockade of the COX metabolite-associated endoperoxide 4 receptors (8, 61) had no effect on the pressor response to dynamic hindlimb muscle stretch in freely perfused rats. Collectively, the evidence suggests that in health, the function of the MA channels on thin fiber sensory neurons that underlie dynamic mechanoreflex activation are not under the basal influence of COX metabolite receptor or IP_3 receptor mediated signaling.

Our finding in ligated rats that IP_3 receptor blockade with XeC reduced the pressor and cardioaccelerator response to stretch identifies a second messenger signaling component through which TxA_2 receptor-mediated chronic sensitization of MA channels and the mechanoreflex is realized in this simulated PAD model (61). Additionally, in ligated rats we found that the effect of XeC on the pressor response evoked during isolated mechanoreflex activation extends to dynamic muscle contraction. That finding suggests that IP_3 receptors contribute to the sensitization of the MA channels that underlie mechanoreflex activation during rhythmic contraction. The mechanism by which TxA_2 and IP_3 receptor signaling is amplified by chronic femoral artery ligation in the rat includes a functional elevation in TxA_2 receptor protein expression in sensory neurons (10, 61). An increase in sensory neuron IP_3 receptor expression or phosphorylation may also contribute and such a determination is an important future direction. It is also important to note that other Gq protein coupled receptors in addition to TxA_2 receptors, such as bradykinin 2 receptors (43), are also likely to influence IP_3 receptor signaling in ligated rats. The lack of off-target effect of XeC in our experiments, such as a generalized desensitization of sensory neurons produced by altering sodium handling, for example, is supported by the finding that XeC did not reduce the pressor response to the hindlimb arterial

injection of lactic acid. Thus, XeC likely permeated sensory neuron cell membranes, blocked/inhibited IP_3 receptors, and reduced cytosolic calcium concentration within the endings of the sensory neurons that mediate dynamic mechanoreflex activation in ligated rats.

To provide additional evidence that IP_3 receptors contribute to the chronic MA channel and mechanoreflex sensitization in ligated rats, we investigated the effect of a different IP_3 receptor antagonist, namely 2-APB, on the pressor response to dynamic hindlimb muscle stretch. Our finding that 2-APB reduced the pressor response to stretch in ligated rats is consistent with our XeC findings. However, 2-APB is also a TRPV 1, 2, and 3 agonist (13, 30). The substantial pressor response observed in response to the injection of 2-APB into the arterial supply of the hindlimb caught our attention because TRPV1 channel stimulation with capsaicin injected into the hindlimb produces robust pressor responses in decerebrate rats (e.g. (69, 70)), and TRPV1 channel stimulation was found to reduce MA piezo channel function in isolated mouse DRG neurons (6). This prompted us to investigate the effect of TRPV1 channel stimulation with capsaicin on the mechanoreflex. Our finding that the injection of capsaicin into the arterial supply of the hindlimb reduced the pressor response to hindlimb muscle stretch in ligated rats indicates that the effect of 2-ABP on the mechanoreflex may not be restricted to its blockade of IP_3 receptors and may also include its agonism of TRP channels.

We were initially surprised at the marked effect of TRPV1 stimulation with capsaicin on the mechanoreflex given the rigorous experimental evidence in the cat that group III muscle afferents are primarily mechanically sensitive whereas group IV muscle afferents are primarily chemically (including capsaicin) sensitive (36). However, recent investigations have reported co-expression of TRPV1 and MA piezo channels ranging from 24-80% in different populations of mouse DRG neurons (16, 77). The mechanism by which TRPV1 channel stimulation reduced MA channel function in DRG neurons was found to include marked elevations in calcium, activation of protein kinase C (PKC), and a depletion of membrane phosphoinositides (6). Thus, the collective findings of the present investigation suggest a paradoxical relationship where modest increases in sensory neuron cytosolic calcium levels resulting from increased IP_3 receptor signaling augments the mechanoreflex whereas larger increases in cytosolic calcium resulting from TRPV1 stimulation reduces the mechanoreflex.

Several experimental considerations warrant discussion. First, atherosclerosis develops slowly with a gradual narrowing of the arteries in PAD patients whereas the rat model of

simulated PAD relies on instantaneous and complete femoral artery ligation. Nevertheless, femoral artery ligation followed by 72 hours of recovery replicates the limb blood flow patterns during exercise and exaggerated exercise pressor reflex found in PAD patients (76, 81). Second, we did not perform experiments in which daltroban was injected systemically. However, we found recently that the systemic (i.v.) injection of daltroban had no effect on the pressor response to mechanoreflex activation in ligated rats (61) or rats with heart failure (11). Based on those findings, we believe daltroban was unlikely to have systemic effects during contraction in the present investigation. Third, hindlimb muscle stretch stimulates MA channels by lengthening the muscle whereas MA activated channels are stimulated by muscle shortening concurrent with increased intramuscular pressure during muscle contraction (26). Nevertheless, multiple studies, including the present investigation, have found that mechanisms that modulate MA channel function during hindlimb muscle stretch maneuvers in decerebrate rats also modulate the pressor response to hindlimb muscle contraction (e.g., 20, 39, 66, 80). Fourth, baseline MAP and HR were significantly higher in the post-capsaicin dynamic stretch condition. However, we do not believe that influenced the pressor response as the findings are similar to the effect of 2-ABP on the pressor response to dynamic stretch. Lastly, investigating the specific downstream mechanism(s) by which IP_3 receptor signaling modulates MA channel function was beyond the scope of this investigation but constitutes an important future direction. For example, activation of protein kinases (32, 47) and and/or modification of the cytoskeleton (19) resulting in altered gating properties of MA channels may be involved.

In summary, we investigated important extensions of our previous findings that COX metabolite (10) and TxA_2 receptor (61) signaling contributes to a chronic sensitization of MA channels and the mechanoreflex in rats with a ligated femoral artery (38). We first confirmed that the role for TxA_2 receptors in chronic mechanoreflex sensitization in ligated rats recently reported by our laboratory is reflected when the exercise pressor reflex is generated during skeletal muscle contraction. We also found that IP_3 receptor signaling, a component of the second messenger signaling linked to G_q protein coupled receptors such as TxA_2 receptors, contributed importantly to the chronic mechanoreflex sensitization in rats with a ligated femoral artery. PAD patients experience exaggerated increases in blood pressure during exercise and reduced exercise tolerance compared to healthy counterparts (4, 5, 42, 44, 52, 55, 56). This investigation reveals important mechanisms within thin fiber sensory neurons that may

contribute to reflex-mediated sympathoexcitation and exaggerated blood pressure increases during exercise in this patient population.

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Table 3.1 ***Baseline mean arterial pressure (MAP) and heart rate (HR)***

Baseline MAP (mmHg)

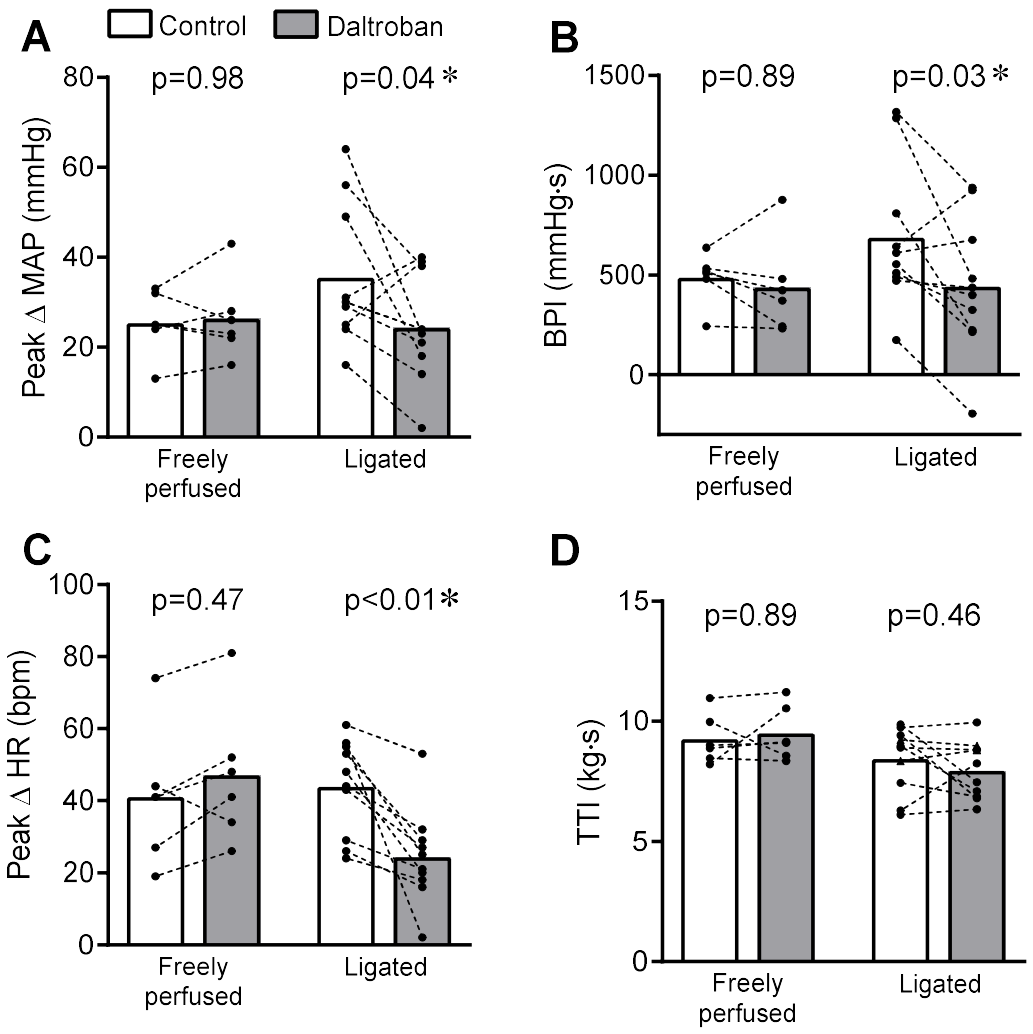
Experimental group	Control	Post condition	p-value
Freely perfused contraction (TxA_2-R antagonist, n=6)	107±8	108±10	0.68
Ligated contraction (TxA_2-R antagonist, n=10)	98±6	108±6	0.11
Ligated contraction (1% DMSO, n=5)	99±12	101±10	0.76
Freely perfused stretch (IP_3-R antagonist XeC, n=9)	128±5	121±4	0.26
Ligated stretch (IP_3-R antagonist XeC, n=14)	113±5	119±5	0.21
Ligated stretch (IP_3-R antagonist XeC i.v., n=5)	133±7	132±5	0.81
Ligated stretch (0.02% ethanol, n=6)	109±12	115±13	0.53
Ligated lactic acid inj. (IP_3-R antagonist XeC, n=5)	125±15	118±4	0.58
Ligated contraction (IP_3-R antagonist XeC, n=5)	100±9	101±9	0.91
Freely perfused contraction (IP_3-R antagonist XeC, n=4)	98±13	100±7	0.82
Ligated stretch (IP_3-R antagonist 2APB, n=6)	129±5	127±7	0.83
Ligated stretch (3.75% DMSO, n=4)	118±15	114±12	0.42
Ligated stretch (Capsaicin, n=6)	96±6	114±3	0.03*

Baseline HR (bpm)

Experimental group	Control	Post condition	p-value
Freely perfused contraction (TxA_2-R antagonist, n=6)	391±19	388±22	0.63
Ligated contraction (TxA_2-R antagonist, n=10)	382±13	412±20	0.10
Ligated contraction (1% DMSO, n=5)	430±16	426±21	0.68
Freely perfused stretch (IP_3-R antagonist XeC, n=9)	509±13	517±14	0.13
Ligated stretch (IP_3-R antagonist XeC, n=14)	494±13	507±11	0.11
Ligated stretch (IP_3-R antagonist XeC i.v., n=5)	523±4	516±5	0.32
Ligated stretch (0.02% ethanol, n=6)	517±13	520±13	0.85
Ligated contraction (IP_3-R antagonist XeC, n=5)	398±19	389±16	0.31
Freely perfused contraction (IP_3-R antagonist XeC, n=4)	344±7	357±13	0.47
Ligated lactic acid inj. (IP_3-R antagonist XeC, n=5)	544±8	550±8	0.26
Ligated stretch (IP_3-R antagonist 2APB, n=6)	497±12	513±10	0.10
Ligated stretch (3.75% DMSO, n=4)	490±11	502±11	0.04*
Ligated stretch (Capsaicin, n=6)	465±17	495±9	0.03*

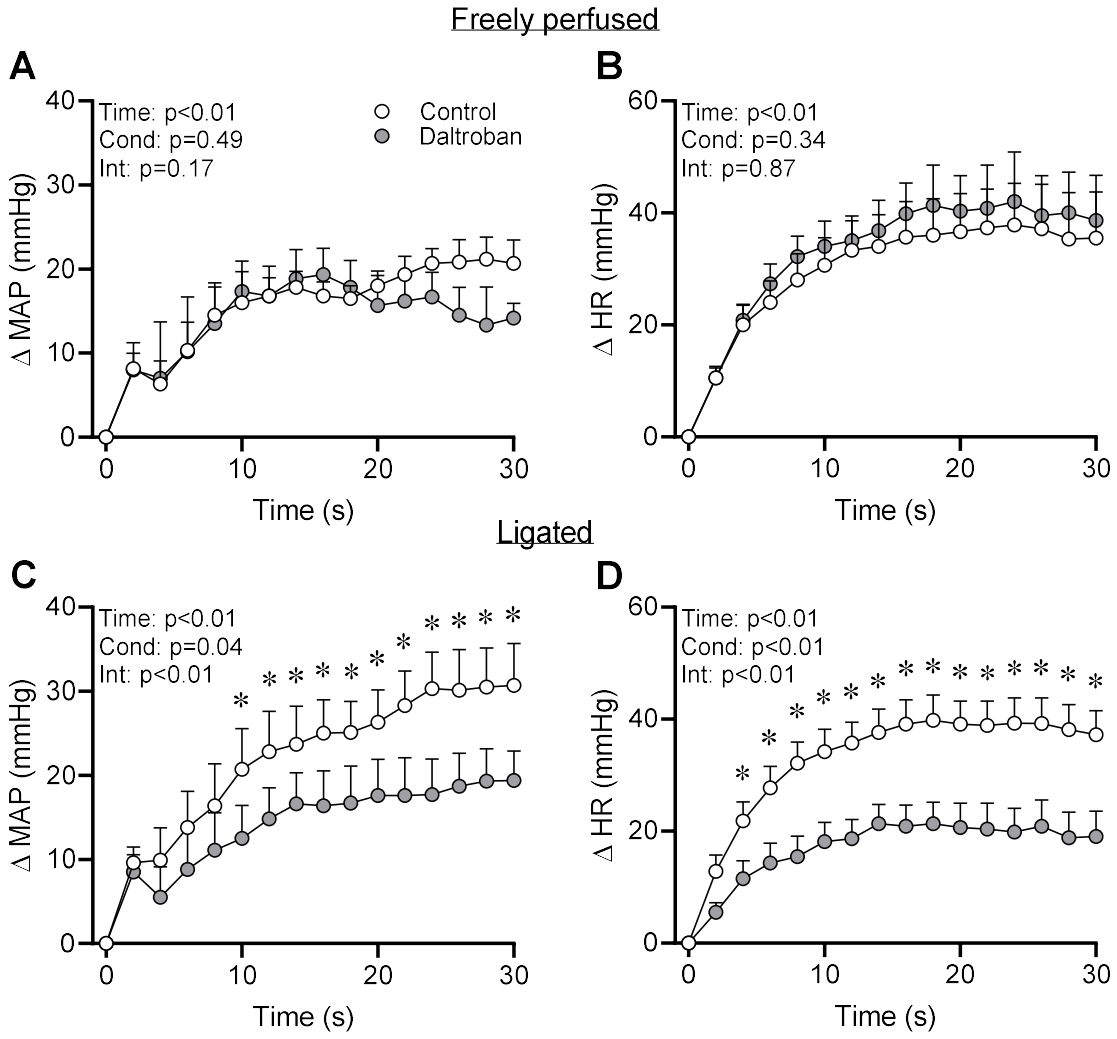
The experimental group is identified by the presence of a ligated or patent (freely perfused) femoral artery, the experimental maneuver, and the injectate. Values are mean±SEM. TxA_2-R, thromboxane A_2 receptor. IP_3-R, inositol-1-4-5-trisphosphate receptor. Data were analyzed with paired Student's t-tests. Asterisks indicate statistical significance ($p \leq 0.05$).

Figure 3.1 ***Effect of thromboxane A_2 (TxA_2) receptor blockade with daltroban on the exercise pressor reflex***



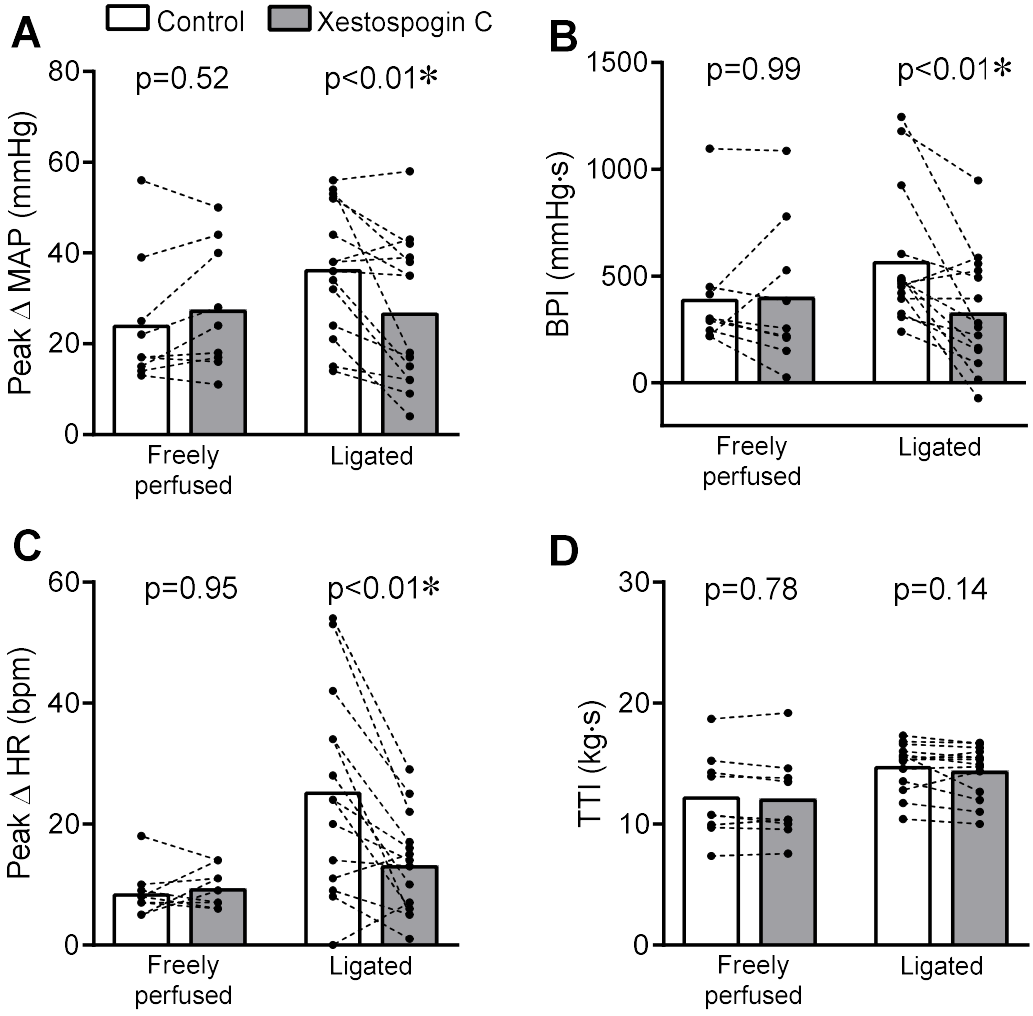
The peak Δ mean arterial pressure (peak Δ MAP, A), blood pressure index (BPI, B), and peak Δ heart rate (peak Δ HR, C) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle contraction before (Ctrl) and after injection of the TxA_2 receptor antagonist daltroban (80 μg) into the arterial supply of the hindlimb of freely perfused (n=6) and ligated (n=10) rats. The tension-time index (Δ TTI, D) was not different between conditions for either group. Data were analyzed with Sidak multiple comparisons tests. Bars represent group mean. Asterisks indicate statistically significant ($p \leq 0.05$) differences between conditions.

Figure 3.2 ***Effect of thromboxane A_2 (TxA_2) receptor blockade with daltroban on the time course of the exercise pressor reflex***



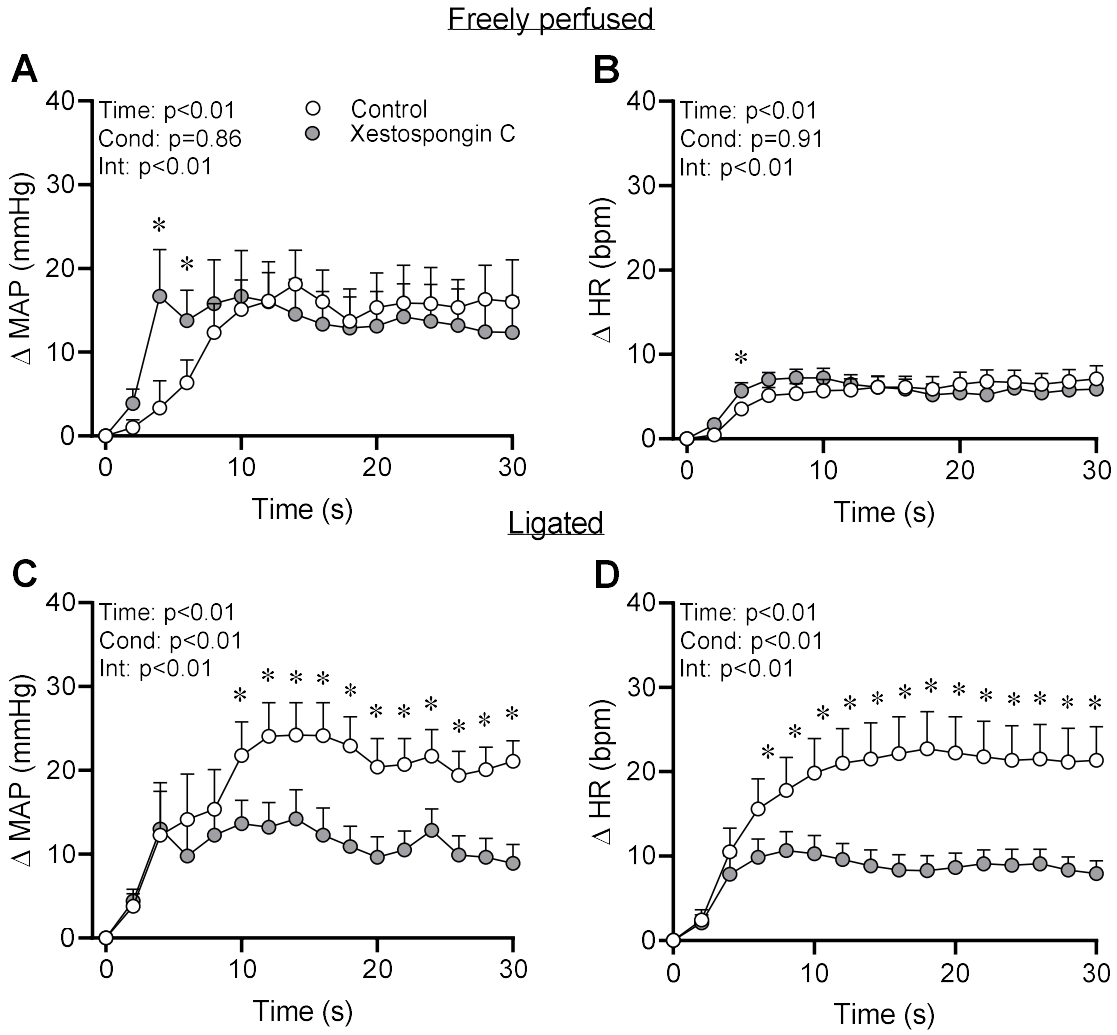
The Δ mean arterial pressure (Δ MAP, A and C) and Δ heart rate (Δ HR, B and D) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle contraction before and after injection of TxA_2 receptor antagonist daltroban (80 μg) into the arterial supply of the hindlimb of freely perfused (top panels, n=6) and ligated (bottom panels, n=10) rats. Data were analyzed with two-way repeated measures ANOVA and Sidak multiple comparisons tests. Asterisks indicate time points where statistically significant ($p \leq 0.05$) differences exist between conditions. Cond. = condition effect, Int. = interaction.

Figure 3.3 ***Effect of inositol 1-4-5-trisphosphate (IP_3) receptor blockade with xestospongin C (XeC) on isolated mechanoreflex activation***



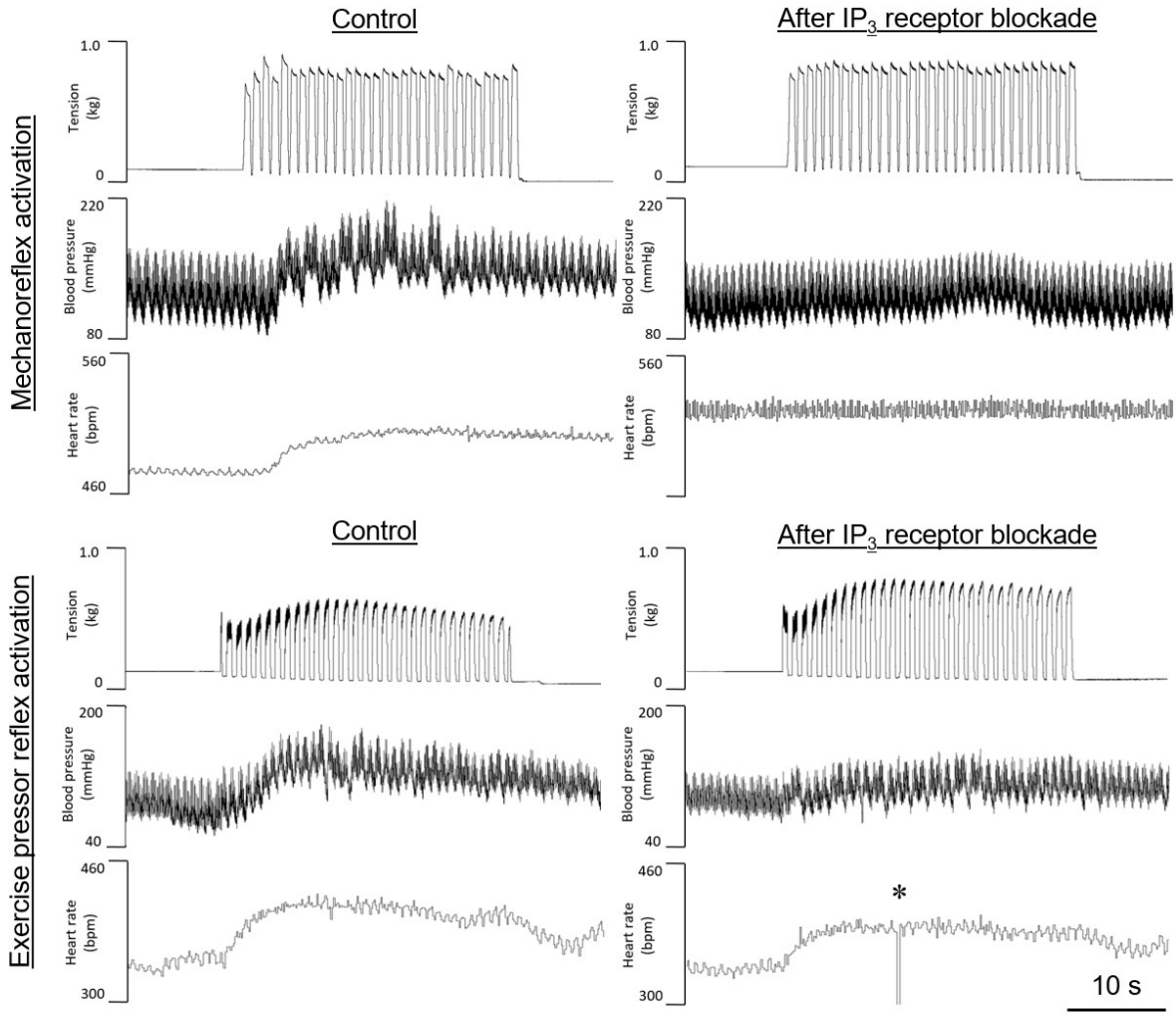
The peak Δ mean arterial pressure (peak Δ MAP, A), blood pressure index (BPI, B), and peak Δ heart rate (peak Δ HR, C) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle stretch before (Ctrl) and after injection of the IP_3 receptor antagonist XeC (5 μg) into the arterial supply of the hindlimb of freely perfused (n=9) and ligated (n=14) rats. The tension-time index (Δ TTI, D) was not different between conditions for either group. Data were analyzed with Sidak multiple comparisons tests. Bars represent group mean. Asterisks indicate statistically significant ($p \leq 0.05$) differences between conditions.

Figure 3.4 ***Effect of inositol 1-4-5-trisphosphate (IP_3) receptor blockade with xestospongin C (XeC) on the time course of isolated mechanoreflex activation***



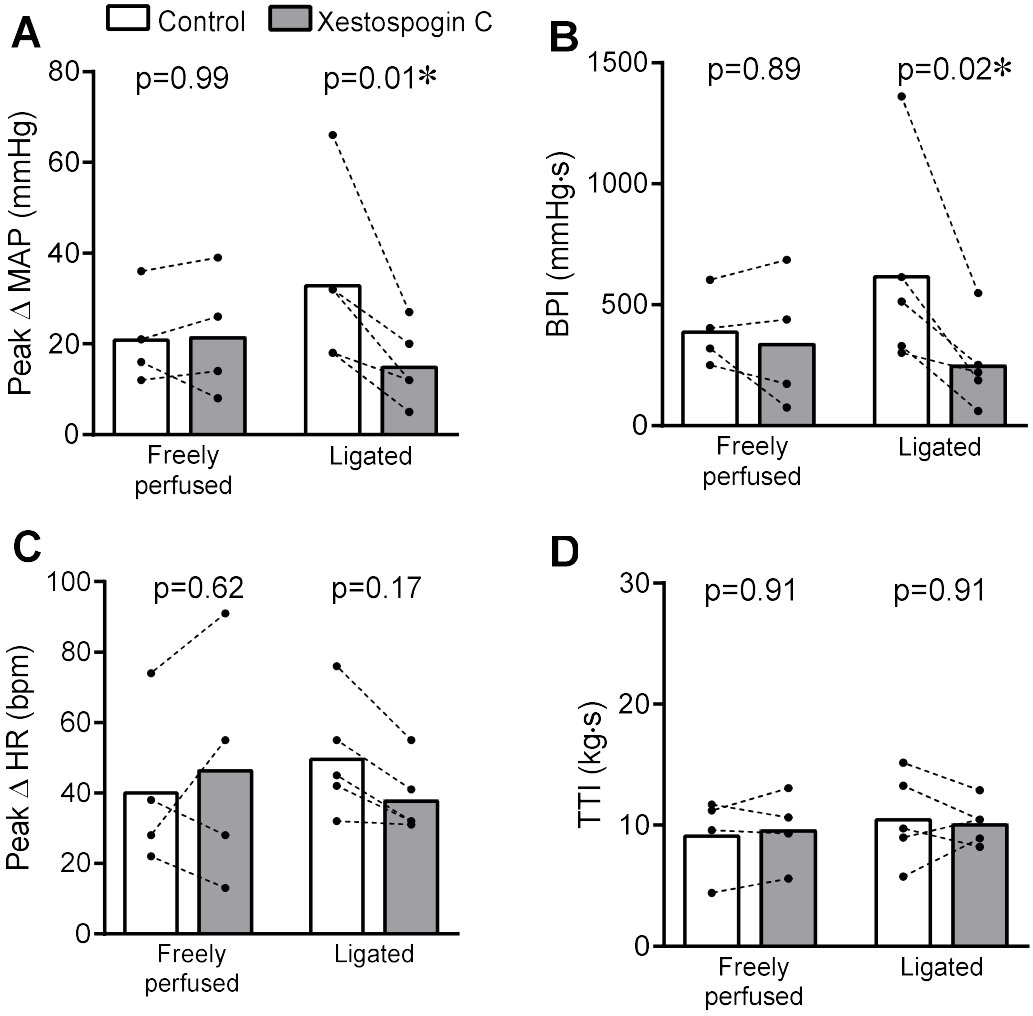
The Δ mean arterial pressure (Δ MAP, A and C) and Δ heart rate (Δ HR, B and D) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle stretch before and after injection of the IP_3 receptor antagonist XeC (5 μg) into the arterial supply of the hindlimb of freely perfused (top panels, n=9) and ligated (bottom panels, n=14) rats. Data were analyzed with two-way repeated measures ANOVA and Sidak multiple comparisons tests. Asterisks indicate time points where statistically significant ($p \leq 0.05$) differences exist between conditions. Cond. = condition effect, Int. = interaction.

Figure 3.5 ***Original data tracings of dynamic stretch and contraction before and after IP_3 receptor blockade***



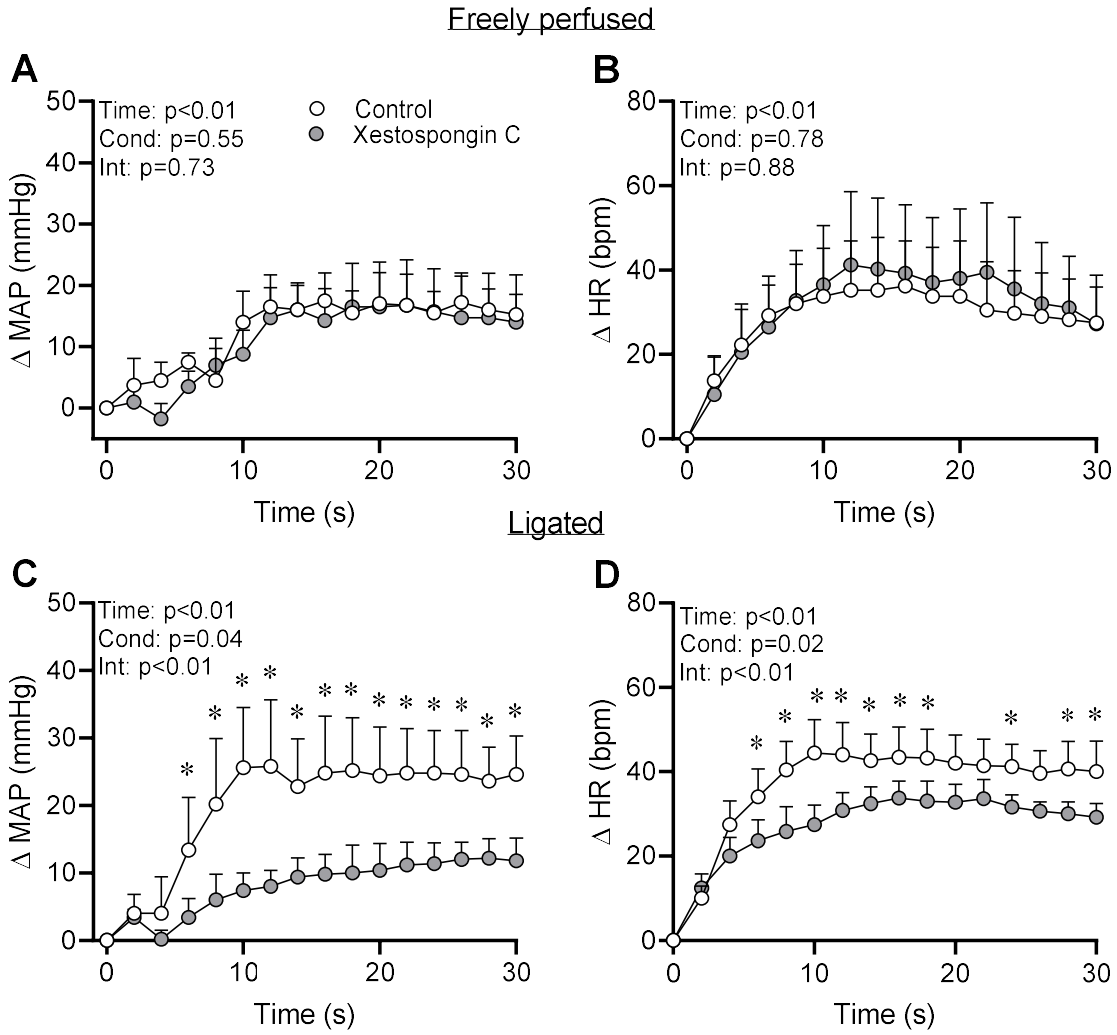
Top panels: Original tracings in ligated rats of the blood pressure and heart rate response to 30 s of 1 Hz dynamic hindlimb skeletal muscle stretch before (*left*) and after (*right*) injection of inositol 1-4-5-trisphosphate (IP_3) receptor antagonist xestospongin C (5 μg) into the arterial supply of the hindlimb. *Bottom panels:* Original tracings in a ligated rat of the blood pressure and heart rate response to 30 s of 1 Hz dynamic hindlimb skeletal muscle contraction before (*left*) and after (*right*) injection of xestospongin C (5 μg) into the arterial supply of the hindlimb. The asterisk denotes artifact in the HR signal that was excluded from analysis.

Figure 3.6 ***Effect of inositol 1-4-5-trisphosphate (IP_3) receptor blockade with xestospongin C on the exercise pressor reflex***



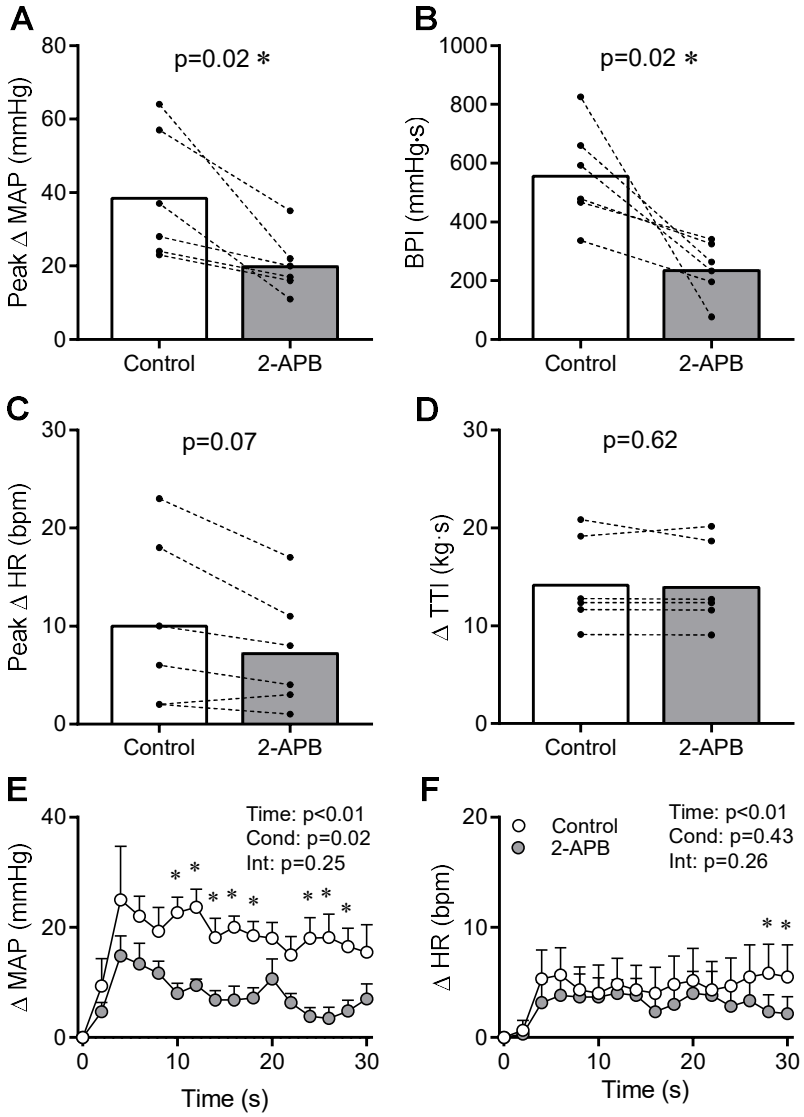
The peak Δ mean arterial pressure (peak Δ MAP, A), blood pressure index (BPI, B), and peak Δ heart rate (peak Δ HR, C) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle contraction before (Ctrl) and after injection of the IP_3 receptor antagonist XeC (5 μg) into the arterial supply of the hindlimb of freely perfused (n=4) and ligated rats (n=5). The tension-time index (Δ TTI, D) was not different between conditions. Data were analyzed with Sidak multiple comparisons tests. Bars represent group mean. Asterisks indicate statistically significant ($p \leq 0.05$) differences between conditions.

Figure 3.7 ***Effect of inositol 1-4-5-trisphosphate (IP_3) receptor blockade with xestospongin C (XeC) on the time course of the exercise pressor reflex***



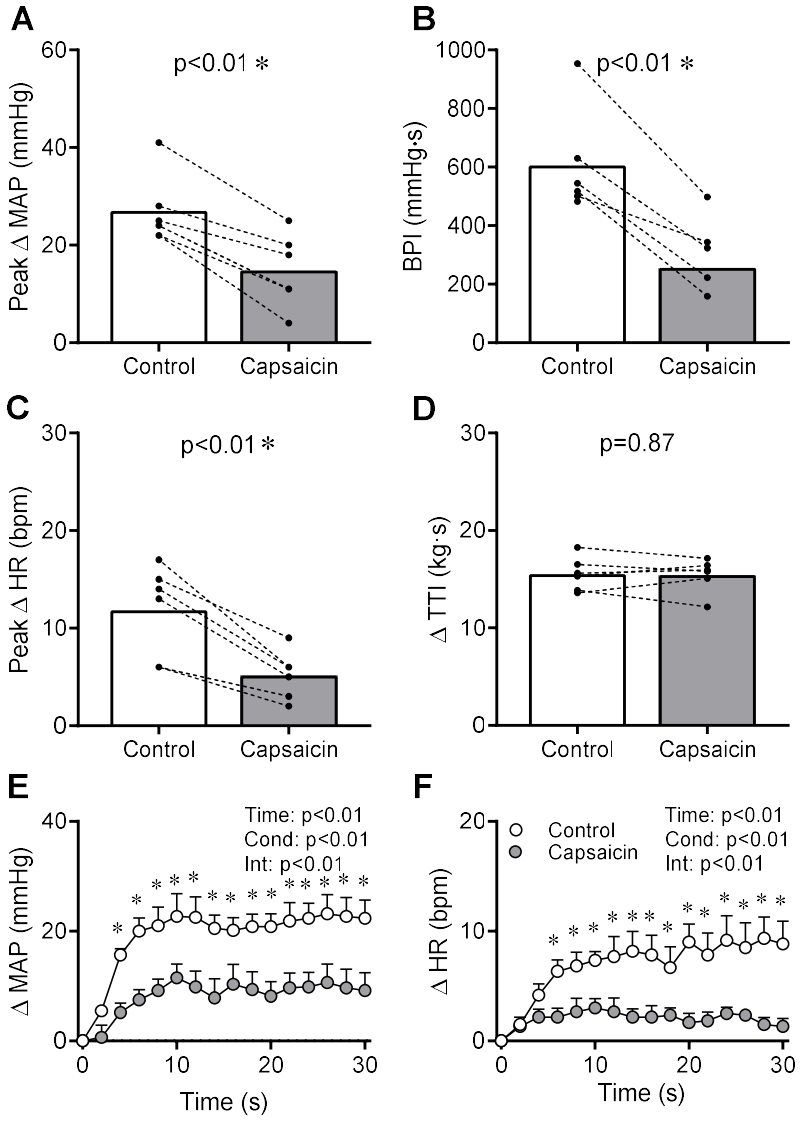
The Δ mean arterial pressure (Δ MAP, A and C) and Δ heart rate (Δ HR, B and D) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle contraction before and after injection of the IP_3 receptor antagonist XeC (5 μg) into the arterial supply of the hindlimb of freely perfused (top panels, n=4) and ligated (bottom panels, n=5) rats. Data were analyzed with two-way repeated measures ANOVA and Sidak multiple comparisons tests. Asterisks indicate time points where statistically significant ($p \leq 0.05$) differences exist between conditions. Cond. = condition effect, Int. = interaction.

Figure 3.8 ***Effect of 2-ABP on isolated mechanoreflex activation***



The peak Δ mean arterial pressure (Δ MAP, A), blood pressure index (BPI, B), peak Δ heart rate (ΔHR, C), and MAP and HR time course (E and F) response to 30 s 1 Hz dynamic hindlimb skeletal muscle stretch before and after injection of 2-APB (170 µg) into the arterial supply of the hindlimb of ligated rats (n=6). The tension-time index (Δ TTI, D) was not different between conditions Data are analyzed with paired Student's t-tests (A-D) or two-way repeated measures ANOVA with Sidak multiple comparisons tests (E and F). Bars are group mean. Asterisks indicate statistically significant ($p \leq 0.05$) differences between conditions. Cond. = condition effect, Int. = interaction.

Figure 3.9 ***Effect of capsaicin on isolated mechanoreflex activation***



The peak Δ mean arterial pressure (Δ MAP, A), blood pressure index (BPI, B), peak Δ heart rate (ΔHR, C), and MAP and HR time course (E and F) response to 30 s 1 Hz dynamic hindlimb skeletal muscle stretch before and after injection of capsaicin (0.5 µg) into the arterial supply of the hindlimb of ligated rats (n=7). The tension-time index (Δ TTI, D) was not different between conditions Data are analyzed with paired Student's t-tests (A-D) or two-way repeated measures ANOVA with Sidak multiple comparisons tests (E and F). Bars are group mean. Asterisks indicate statistically significant ($p \leq 0.05$) differences between conditions. Cond. = condition effect, Int. = interaction.

Chapter 4 - Actin Polymerization Inhibition Reduces the Mechanoreflex in Rats with Simulated Peripheral Artery Disease

Abstract

Mechanically-activated (MA) channels on group III/IV skeletal muscle afferent sensory endings are stimulated during muscle contraction which initiates autonomic and cardiovascular adjustments (the mechanoreflex). The mechanoreflex is one component of the exercise pressor reflex, the activation of which, in health, supports exercise performance. Accumulating evidence indicates that in peripheral artery disease (PAD), cellular signaling mechanisms within sensory neurons sensitize MA channels and exaggerate the mechanoreflex which increases cardiovascular risk. MA channels are tethered to the cytoskeleton and cellular signaling-induced cytoskeleton alterations have been shown to augment MA channel function. Here we investigated whether polymerization of actin filaments in sensory neurons contributes to the exaggerated mechanoreflex and exercise pressor reflex in a rat model of simulated PAD in which a femoral artery is ligated for 72 hours. We hypothesized that injection of cytochalasin D (actin polymerization inhibitor) into the hindlimb arterial supply would reduce the pressor response to 30 seconds of 1 Hz repetitive/dynamic hindlimb skeletal muscle stretch (model of mechanoreflex activation) and contraction (model of exercise pressor reflex activation) in decerebrate, unanesthetized rats with a ligated femoral artery. We found that cytochalasin D reduced the pressor response to skeletal muscle stretch and contraction in “ligated” rats but not in rats with freely perfused hindlimb muscles. Pre-treatment with the actin stabilizer phalloidin markedly attenuated the effect of cytochalasin D in ligated rats. In conclusion, in rats with a ligated femoral artery, actin polymerization within sensory neurons augments MA channel sensitivity which contributes to the exaggerated mechanoreflex and exercise pressor reflex.

Introduction

Mechanically-activated (MA) channels located on group III and IV skeletal muscle afferents stimulated during skeletal muscle contraction contribute to reflex increases in sympathetic nervous system activity and subsequent increases in heart rate and blood pressure to support skeletal muscle perfusion (21, 22, 25, 26). This reflex, termed the mechanoreflex, along with its metabolic counterpart, comprise the exercise pressor reflex (20, 27, 39). The mechanoreflex is necessary for appropriate cardiovascular responses to exercise in health, but contributes to sympathoexcitation and exaggerated increases in blood pressure in peripheral artery disease (PAD). Specifically, the mechanoreflex was found to contribute to the exaggerated pressor response during dynamic low intensity plantar flexion exercise in patients with PAD compared to age-matched healthy controls (28, 29). Likewise, the mechanoreflex was also found to be exaggerated during 1 Hz dynamic hindlimb muscle contraction in a rat model of simulated PAD in which the femoral artery was ~72 hr ligated compared to the hindlimb in which the femoral artery remained patent (6, 23).

A recently discovered class of MA channel named "piezo" is believed to underlie mechanoreflex activation (5, 31). Specifically, our laboratory (31) and others (5, 6, 13, 14) have used GsMTx4, tarantula toxin that inhibits piezo channels with selectively over other classes of MA channels including TRPC6 (13), to reveal that piezo channels contribute to the generation of the mechanoreflex and the exercise pressor reflex in healthy rats (5, 13, 31), rats with type I diabetes (13, 14) and in the rat model of simulated PAD in which a femoral artery is ligated for 72 hours (6). Specifically, in rats with a ligated femoral artery, GsMTx4 was found to reduce the pressor response to 1 Hz dynamic hindlimb skeletal muscle contraction to a greater extent than that found in rats with patent femoral arteries (5). This enhanced role for piezo channels was found despite no effect of femoral artery ligation on piezo channel protein expression in lumbar dorsal root ganglia (DRG; cell bodies of sensory endings(6)). Thus, a sensitization of piezo channels, likely attributable to upregulations of cellular signaling pathways within sensory neuron endings (11, 12), appears to account for enhanced piezo channel function in rats with ligated femoral arteries. For example, we found recently that blockade of cyclooxygenase metabolite linked thromboxane A_2 (TxA_2) receptors (30), as well as blockade of inositol-1,4,5 trisphosphate (IP_3) receptors (a component of the intracellular signaling pathway linked to TxA_2 receptors) reduced the pressor response a repetitive 1 Hz hindlimb skeletal muscle stretch

protocol in rats with a ligated femoral artery but not in rats with patent femoral arteries. The hindlimb muscle stretch protocol serves as a model of mechanoreflex activation isolated from contraction induced metabolic signals. Although our recent findings identified signaling pathways linked to mechanoreflex, and presumably piezo channel, sensitization in rats with a ligated femoral artery but the specific mechanism by which the function of the channels are directly impacted remain elusive.

The majority of previous studies, both *in vitro* and *in vivo*, have focused on biochemical aspects of MA channel regulation including modulation by cellular signaling pathways. Several lines of experimental evidence also suggest an important role for biophysical aspects of MA channel regulation including modulation by structural components of the cell cytoskeleton. The cytoskeleton is an intricate, dynamic network of interlinking proteins that include actin filaments, microtubules, and intermediate filaments along with their associated proteins below the surface of the plasma membrane. Importantly, upregulated intracellular signaling can impact actin polymerization and contributes to abnormal pain related behaviors (15). Piezo channels have been found to be functionally tethered to the actin cytoskeleton via the E-cadherin, β-catenin, and vinculin mechanotransduction complex and disruption of the intermolecular interaction prevents actin-dependent piezo channel gating of MA (42). Furthermore, actin polymerization can deform the plasma membrane and increase static plasma membrane tension (33) which has been shown to impact the gating properties of piezo channels. For example, increased static membrane tension induced by osmosis/cell swelling in primary afferent dorsal root ganglia (DRG) neurons was found to potentiate piezo channel-mediated currents (17). Cytochalasin D (CD), a potent cell permeable inhibitor of actin polymerization, significantly decreased DRG neuron membrane tension and reduced the potentiated piezo-mediated currents (17, 18). Inhibition of actin polymerization with CD has also been shown to prevent mechanical hyperalgesia in a mouse model of chronic pain in which inflammatory second messenger signaling is dependent on an intact cytoskeleton (8). Whether cell-signaling induced alterations in the cytoskeleton, especially actin polymerization, in thin fiber skeletal muscle afferents contributes to chronic mechanoreflex sensitization in cardiovascular disease remains unknown.

The purpose of the present study was to investigate whether actin polymerization within sensory neurons enhances the sensitivity of MA channels on thin fiber muscle afferents and therefore contributes to the chronic mechanoreflex sensitization observed in rats with a ligated

femoral artery. We tested the hypothesis that injection of the actin polymerization inhibitor CD into the arterial supply of the hindlimb reduces the pressor and cardioaccelerator response to 30 seconds of 1 Hz dynamic hindlimb skeletal muscle stretch to a greater extent in rats with a ligated femoral artery than in sham-operated rats with freely perfused hindlimb muscles.

Methods and Materials

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 (~12-15 weeks old) male Sprague-Dawley rats (n=39, average body weight: 403±10 g; Charles River Laboratories). The rats were housed two per cage in temperature (maintained at 22°C) and light (12-12 hr light-dark cycle)-controlled accredited facilities with standard rat chow and water provided *ad libitum*. At the end of each experiment, the decerebrated rats (see below) were killed by an intravenous injection of saturated (>3 mg/kg) potassium chloride.

Femoral artery ligation/sham procedure. Of the 39 rats in this investigation, 34 had their left femoral artery ligated ~72 hrs before the terminal experimental protocol was performed. Briefly, rats were anesthetized with 2% isoflurane anesthesia (balance O_2) and their left femoral artery was surgically exposed and ligated tightly with 6-0 silk suture ~3–5 mm distal to the inguinal ligament. In 5 rats, a sham ligation procedure was performed in which the left femoral artery was surgically exposed, and 6-0 suture was passed under the femoral artery but not tied. Thus, the hindlimb remained freely perfused following the sham procedure. In both "ligated" and "freely perfused" rats (referred to as such from here forward for simplicity), the incisions were closed, meloxicam was administered (1-2 mg/kg s.c.) as an analgesic, and the rats were monitored daily until the final experiment.

Surgical procedures for experimental protocols. On the day of the experiment, rats were anesthetized with ~2% isoflurane (balance O_2). Adequate depth of anesthesia was confirmed by the absence of toe-pinch and blink reflexes. The trachea was cannulated, and the lungs were mechanically ventilated (Harvard Apparatus model 683) with the gaseous anesthetic until the decerebration was completed (see below). In all rats, the right jugular vein and both common carotid arteries were cannulated with PE-50 catheters for the administration of fluids/drugs, measurement of arterial blood pressure (Physiological Pressure Transducer, AD Instruments), and sampling of arterial blood gases (ABL80 Flex, Radiometer). Heart rate (HR) was measured by electrocardiogram. The left calcaneus bone was severed and the triceps surae (gastrocnemius, soleus and plantaris) muscles were exposed by reflecting the overlying skin and skeletal muscles. A string was then tied to the distal Achilles tendon and severed calcaneus which linked the

triceps surae muscles to a force transducer (Grass FT03) and rack and pinion that could be turned manually. In the 5 sham rats and 31 of the ligated rats, the left superficial epigastric artery was cannulated with a PE-8 catheter whose tip was located near the junction of the superficial epigastric artery and the femoral artery. In those rats in which a catheter was placed in the left superficial epigastric artery, a reversible snare was placed around the left iliac artery and vein (i.e., proximal to the location of the catheter placed in the superficial epigastric artery). For rats in which dynamic hindlimb skeletal muscle contraction was performed (n=5 ligated rats), the left sciatic nerve was exposed.

After the initial surgical procedures, all rats were placed in a Kopf stereotaxic frame with clamps placed around the pelvis. Dexamethasone (0.2 mg i.v.) was injected to minimize brainstem edema. A precollicular decerebration was performed and all neural tissue rostral to the superior colliculus was aspirated. After the decerebration was completed, anesthesia was terminated and the lungs were mechanically ventilated with room air. The decerebration procedure was performed because anesthesia has been shown to depress the exercise pressor reflex in the rat (34). Arterial blood gases and pH were measured periodically with a blood gas analyzer and maintained within normal limits ($PaCO_2$: 35–45 mmHg, PaO_2: ~100 mmHg, pH: 7.35–7.45) by adjusting ventilation and/or administering intravenous sodium bicarbonate (8.5%). Core temperature was measured by a rectal probe and maintained at ~37-38°C by an automated heating system (Harvard Apparatus) and heat lamp. For all rats in which dynamic hindlimb muscle stretch was performed (27 of the 39 rats) the paralytic pancuronium bromide (1 mg/kg i.v.) was injected prior to the initiation of any stretch maneuver in order to prevent any spontaneous or reflex muscle contraction which would produce a metabolic stimulus.

Isolated mechanoreflex activation protocol. To study mechanoreflex activation in isolation from contraction-induced metabolite production, our laboratory uses a 1 Hz dynamic hindlimb skeletal muscle stretch adapted by the work by Stebbins and colleagues (7, 35). Dynamic stretch of a hindlimb in which the femoral artery was previously ~72 hr ligated evokes larger sympathetic nervous system activity and blood pressure responses than that evoked was a hindlimb in which the femoral artery remained patent (2, 23, 30, 31).

The control dynamic hindlimb muscle stretch maneuver was performed at least 60 minutes following termination of isoflurane anesthesia. To begin, baseline muscle tension was set at ~100 g and baseline MAP and HR were collected for 30 seconds. An experienced

investigator then elicited repetitive/dynamic triceps suare muscle stretch for 30 seconds by manually turning the rack and pinion at a 1 Hz frequency with the aid of a metronome. The investigator aimed to develop ~0.6 to 0.8 kg of tension during each dynamic stretch maneuver because that is the tension typically developed during hindlimb muscle contractions in decerebrate rat preparations (5, 6, 23). Moreover, the investigator aimed for consistent levels of tension development for each individual dynamic stretch although slight variability in tension development was often present. The dynamic stretch protocol was adapted from that described by Daniels et. al. (7). ~5 minutes following the control stretch maneuver, the snare on the left iliac artery and vein was tightened and 400 ng of CD (dissolved in 0.4 ml of 0.025% DMSO, n=5 freely perfused rats and n=7 ligated rats) or 0.4 ml of a 0.025% DMSO solution alone (the vehicle for CD, n=3 ligated rats) was injected into the arterial supply of the hindlimb via the left superficial epigastric artery catheter. After 10 minutes, the left iliac artery and vein snare was released and the hindlimb was allowed to reperfuse for 20 minutes before the dynamic stretch protocol was repeated as described above. At the end of each experiment, Evans blue dye was injected in the same manner as the experimental solution to confirm that the injectate had access to the triceps surae muscle circulation.

In an additional group of four ligated rats, dynamic hindlimb skeletal muscle stretch maneuvers were performed before and after CD (400 ng) was injected into the right jugular vein and therefore allowed to circulate systemically. Thirty minutes elapsed between the i.v. injection of CD (400 ng) and the subsequent stretch maneuver exactly as described above in protocol 1 when CD was injected into the arterial supply of the hindlimb.

Lactic acid and α,β-methylene ATP injection protocols. In separate groups of ligated rats, an injection of either 0.2 ml of 24 mM lactic acid (n=4) or 10 µg α,β-methylene ATP (n=4) into the arterial supply of the hindlimb was performed before and after CD (400 ng) was injected into the arterial supply of the hindlimb exactly as described above in the isolated mechanoreflex activation protocol. Thirty minutes elapsed between the injection of the CD and the subsequent lactic acid injection maneuver also as described above.

Phalloidin experiments. In nine ligated rats, the actin stabilizer phalloidin (actin stabilizer, 0.4ml) was injected into the arterial supply of the hindlimb following the decerebration procedure and 1 hr before the control dynamic stretch maneuver. Specifically, either 10 µg (n=5) or 20 µg (n=4) of phalloidin was injected into the arterial supply of the

hindlimb as described above where it remained trapped within hindlimb circulation for five minutes. The hindlimb was then reperfused for 55 minutes at which point the effect of 400 ng of CD on the mechanoreflex was determined as described above.

Exercise pressor reflex activation protocol. The control dynamic hindlimb muscle contraction maneuver was performed at least 60 minutes following termination of isoflurane anesthesia. To begin, baseline muscle tension was set to ~100 g and baseline blood pressure and HR were measured for ~30 seconds. The sciatic nerve was then electrically stimulated using stainless steel electrodes for 30 seconds at a voltage of ~1.5x motor threshold (0.01 ms pulse duration, 500 ms train duration, 40 Hz frequency) which produced 1 Hz repetitive/dynamic contractions of the triceps surae muscles. ~10-15 minutes following the control contraction maneuver, the snare on the left iliac artery and vein was tightened and 400 ng of CD was injected into the arterial supply of the hindlimb via the left superficial epigastric artery catheter (n=5 ligated rats). After 10 minutes, the left iliac artery and vein snare was released and the hindlimb was allowed to reperfuse for 20 minutes before the muscle contraction protocol was repeated as described above. At the end of the experiment, to ensure that the increase in blood pressure during contraction was not due to electrical activation of the axons of the thin fiber afferents in the sciatic nerve, we administered the paralytic pancuronium bromide (1 mg/kg i.v.) and the sciatic nerve was stimulated for 30 s with the same parameters as those used to elicit contraction. No increase in blood pressure was observed during the stimulation period after the administration of pancuronium bromide in any of the experiments for which data are reported which indicates the increases in blood pressure during contractions were reflex in nature. Additionally, at the end of each experiment Evans blue dye was injected in the same manner as the experimental solution to confirm that the injectate had access to the triceps surae muscle circulation.

Drugs. CD was dissolved in 100% DMSO and diluted to a final concentration of 400 ng in 0.4 ml of 0.025% DMSO. The selected dose of CD was calculated from *in vitro* molarity concentrations assuming a hindlimb blood volume of ~15 ml (43). 25 nM of CD that was found to inhibit actin polymerization by half (IC_{50}) in dorsal root ganglia (32). We used double the IC_{50} in the present study. Lactic acid was dissolved in saline and diluted to a final concentration of 24 mM. This dose based on previous use of lactic acid to stimulate ASICs (30, 31, 41). α,β-methylene ATP was dissolved in saline and diluted to 10 μg/kg. The dose was based on previous

use of α,β-methylene ATP to stimulate purinergic receptors (38). Phalloidin was dissolved initially in 100% DMSO and diluted to a final concentration of 10 μg in 0.4 ml of 0.025% DMSO or 20 μg in 0.4 ml of 0.05% DMSO. Dose and timing were based on *in vitro* evidence that 1 μg stabilized actin filaments when injected intradermally (8). We increased the dose in the present study since it would be circulating throughout the entire hindlimb.

Data analysis. Data were collected with a PowerLab and LabChart data acquisition system (AD Instruments). Arterial blood pressure, electrocardiogram, and muscle tension were measured, mean arterial pressure (MAP) and HR were calculated, and all data were displayed in real time and recorded for offline analysis. Baseline MAP and HR were determined from the 30 s baseline periods that preceded each maneuver. The peak pressor (peak Δ MAP) and cardioaccelerator (peak Δ HR) responses were calculated as the difference between the peak values wherever they occurred during the 30 second maneuvers and their corresponding baseline value. The tension-time index (TTI) and blood pressure index (BPIs) were calculated by integration of the area under signal during the stretch or contraction maneuver and subtraction of the integrated area under the signal during the corresponding baseline period. The time course of the increase in MAP and HR were plotted as Δ MAP and Δ HR from baseline over the course of the 30 second stretch or contraction maneuvers. Data are expressed as mean±SEM and were analyzed with paired Student's t-tests, two-way repeated measures ANOVAs and/or Sidak multiple comparisons tests as appropriate. Statistical significance was accepted at $p \leq 0.05$.

Results

Effect of the actin polymerization inhibitor CD on isolated mechanoreflex activation. In sham rats (n=5), injection of CD into the arterial supply of the hindlimb had no effect on the pressor or cardioaccelerator response to dynamic hindlimb muscle stretch (Fig. 1 and 2). In contrast, in ligated rats (n=7) CD significantly reduced the pressor and cardioaccelerator response to stretch (Fig. 1 and 2). The Δ TTI of the stretch maneuver was not different between control and CD conditions in either group (Fig. 1). Baseline MAP and HR were not different between conditions for either group (Table 1). An example of an original tracing of the effect of CD during dynamic stretch in a rat in which the femoral artery was previously ligated is shown in the top panels of Fig 3.

In vehicle control experiments in ligated rats (n=3), 0.025% DMSO (the vehicle for CD) had no effect on the peak Δ MAP (control: 69±10, 0.025% DMSO: 68±16 mmHg, p=0.86), BPI (control: 1352±251, 0.025% DMSO: 1325±261 mmHg·s, p=0.91), or peak Δ HR (control: 33±7, 0.025% DMSO: 32±2 bpm, p=0.88) response to stretch. The Δ TTI of the stretch maneuver was not different between control (7.0±0.5 kg·s) and 0.02% ethanol (7.4±0.5 kg·s, p=0.15) conditions. Baseline MAP and HR were not different between conditions (Table 1).

In systemic control experiments in ligated rats (n=4), injection of CD into the jugular vein to allow it to circulate systemically had no effect on the peak Δ MAP (control: 43±10, i.v. CD: 51±14 mmHg, p=0.26), BPI (control: 976±288, i.v. CD: 1071±301 mmHg·s, p=0.20), or peak Δ HR (control: 20±9, i.v. CD: 27±7 bpm, p=0.24) response to stretch. The Δ TTI of the dynamic stretch maneuver was not different between control (10.1±1.0 kg·s) and i.v. CD (10.1±1.2 kg·s, p=0.88) conditions. Baseline MAP and HR were not different between conditions (Table 1). These results suggest that the effect of CD when it was injected into the arterial supply of the hindlimb of ligated rats on the pressor response to stretch is attributable to effects on the sensory endings of thin fiber muscle afferents and not effects elsewhere in the mechanoreflex arc such as the brainstem and/or the spinal cord.

In “off target” control experiments in ligated rats (n=4), injection of CD into the arterial supply of the hindlimb had no effect on the peak Δ MAP (control: 64±9, CD: 57±13 mmHg, p=0.54) or peak Δ HR (control: 26±7, CD: 18±5 bpm, p=0.20) produced in response to the injection of lactic acid into the arterial supply of the hindlimb. In a different group of ligated rats (n=4), injection of CD into the arterial supply of the hindlimb had no effect on the peak Δ MAP

(control: 25±5, CD: 25±5 mmHg, p>0.99) or peak Δ HR (control: 6±2, CD: 9±2 bpm, p=0.13) produced in response to the injection of α,β-methlylene ATP into the arterial supply of the hindlimb. Baseline MAP and HR were not different between conditions in either group (Table 1). These results suggest that the effect of CD when it was injected into the arterial supply of the hindlimb on the pressor response to stretch is most likely attributable to an interruption of the cytoskeletal structural arrangement and MA channels and is not attributable to a local "off-target" effect such as the inhibition of voltage-gated sodium channels.

In experiments investigating whether the effect of CD was specific to the inhibition of actin polymerization, we injected the actin stabilizer phalloidin (10 or 20 μg) into the arterial supply of the hindlimb 1 hr prior to the control stretch maneuver and subsequent injection of CD. With the prior injection of phalloidin, CD still significantly reduced the pressor response to stretch in ligated rats (peak Δ MAP; control: 41±5, post: 33±4 mmHg, p<0.01 and BPI; control: 872±115, post: 660±94 mmHg·s, p<0.01). However, as shown in Fig. 4, the effect of CD on the pressor response to stretch in the presence of phalloidin ("Phalloidin + CD") was significantly attenuated compared to the effect observed with CD alone ("CD", same data reported in Fig. 1). Additionally, as shown in Fig 5., the effectiveness of phalloidin in attenuating the effect of CD occurred in an apparent dose-dependent manner. The Δ TTI of the stretch maneuver was not different between control and CD conditions in either the 10 μg (control: 8.0±0.7, post: 8.3±0.7 kg·s, p=0.21) or 20 μg (control: 6.8±0.1, post: 7.0±0.2 kg·s, p=0.18) phalloidin + CD group. Baseline MAP and HR were not different between conditions (Table 1).

In investigation of whether the isolated mechanoreflex findings extended to experiments evoking the exercise pressor reflex, we found that injection of CD into the arterial supply of the hindlimb of ligated rats (n=5) significantly reduced the pressor, but not the cardioaccelerator, response to contraction (Fig. 5). The Δ TTI of the contraction maneuver was not different between control and CD conditions (Fig. 5D). Baseline MAP and HR were not different between conditions (Table 1). An example of an original tracing of the effect of CD on the cardiovascular responses to dynamic contraction is shown in the bottom panels of Fig. 3.

Discussion

We investigated the role of the cytoskeletal component actin on the exaggerated mechanoreflex and exercise pressor reflex in a rat model of simulated PAD in which the femoral artery was 72 hr ligated. We found that actin polymerization inhibition reduced the pressor response to dynamic hindlimb muscle stretch in ligated, but not freely perfused rats. In ligated rats we extended these findings to dynamic contraction, confirming a role for actin polymerization in the exaggerated exercise pressor reflex. The present investigation provides the first piece of evidence for a biophysical component of sensory neurons that contributes to MA channel sensitization and the exaggerated mechanoreflex in a rat model of simulated PAD.

Multiple pieces of information evidenced the need to investigate biophysical aspects of MA channel modulation and sensitization as it relates to mechanoreflex and exercise pressor reflex activation. Actin has been found to be directly linked to protein kinase Cε (PKCε)-mediated mechanical hyperalgesia in a rat model of chronic pain (8). It has also been shown that activation of Epac-PKC signaling pathway, which occurs in inflammatory conditions, results in greater F-actin expression (*i.e.*, increased actin polymerization), particularly at the perimeter of rat DRG neurons (15). Upregulation of actin polymerization resulting in greater concentrations of F-actin causes changes in biophysical factors of neurons, such as, increased static plasma membrane tension (33). Static plasma membrane tension has been shown to potentiate MA piezo currents, *in vitro*, in primary afferent rat DRG neurons and in HEK293 cells heterologous expressing MA Piezo2 channels and produce mechanical hyperalgesia, *in vivo*, in a rat model of chronic pain (17). MA piezo channels have been found to be linked to actin via a mechanotransduction complex consisting of multiple proteins. Disruption of the mechanotransduction complex that tethers MA piezo channels to actin prevents actin-dependent gating of MA piezo channels (42) and reduces overall mechanical sensitivity of piezo channels (12). It remained unknown whether actin polymerization impacted MA piezo channels responsible for evoking the mechanoreflex and exercise pressor reflex.

Cytochalasin D (CD) is a fungal toxin that binds to the ends of the actin filament and prevents elongation, thus, disrupting/preventing actin polymerization. CD has been used to reduce MA channel currents, *in vitro,* in cultured primary afferent DRG neurons (10, 18) and HEK293 cells heterologous expressing MA Piezo2 (17, 18) and to prevent mechanical hyperalgesia, *in vivo,* by multiple chronic pain models (8, 15, 17). Specifically, CD reduces static

plasma membrane tension which is a biophysical property of cell structure that has been shown to reduce MA piezo channel currents (17). 25 nM of CD is the IC_{50} for actin polymerization inhibition in dorsal root ganglia (32). We used double the IC_{50} (50nM) in the present study. Specifically, 400 ng of CD distributed throughout an estimated hindlimb volume of ~15 ml (43) resulted in a concentration of ~380 ng. Our finding in freely perfused rats that actin polymerization inhibition with CD did not reduce the pressor or cardioaccelerator response to stretch was initially surprising because piezo channels contribute to evoking the mechanoreflex in health and appear to be tethered to actin. However, the data are consistent with findings from Dina et. al. in which CD has no effect on the paw withdrawal threshold (nociceptive measurement) in spite of the fact that CD disrupted the actin, *in vitro*, in DRG neurons and attenuated mechanical hyperalgesia after administration of epinephrine (rat model of chronic pain) (8). Additionally, no single intracellular pathway appears to be responsible for evoking the mechanoreflex in healthy conditions (3, 30). This suggests redundancy among pathways in health (36) and supports the notion that multiple biophysical gating mechanisms are likely responsible for setting the sensitivity of MA channels.

Our finding in ligated rats that actin polymerization inhibition with CD reduced the pressor and cardioaccelerator response to stretch identifies the first structural mechanism that plays a role in the chronic sensitization of MA channels in rats with ligated femoral arteries. Additionally, in ligated rats we found that the effect of CD on the pressor response evoked during isolated mechanoreflex activation extends to dynamic muscle contraction. That finding suggests that actin filaments contribute to the sensitization of the MA channels that underlie mechanoreflex activation during rhythmic contraction. The lack of off-target effect of CD in our experiments, such as a generalized desensitization of sensory neurons, for example, is supported by the finding that CD did not reduce the pressor response to the hindlimb arterial injection of lactic acid or α, β-methylene ATP. Thus, CD likely permeated sensory neuron cell membranes located within the skeletal muscle and inhibited actin polymerization and organization. These findings suggest that actin polymerization within sensory neurons augments MA channel sensitivity which contributes to the exaggerated mechanoreflex in a rat model of simulated PAD.

To provide additional support for the specificity of CD disrupting actin polymerization, we aimed to replicate work from previous studies where cytoskeleton stabilization can protect against the effect of disruptors (8, 19). In the present study, we determined if we could prevent

the CD-induced reduction of the mechanoreflex in ligated rats by prior stabilization of actin with phalloidin. Phalloidin is a toxin isolated from the "death cap" mushroom and binds to F-actin and prevents actin depolymerization. We found that injection of phalloidin intra-arterially into hindlimb circulation prior to injection of CD resulted in a significant reduction in the pressor response, although the attenuating effects of CD appear to be mitigated in the presence of phalloidin. Additionally, the effect of phalloidin appears to be effect to pressor response in a dose-dependent manner. These data provide support for the specificity of CD on actin filaments and the importance of actin polymerization in the sensitization of MA channels activated during dynamic hindlimb skeletal muscle stretch in a rat model of simulated PAD.

Piezo channels are thought to be largely responsible for evoking the mechanoreflex (5, 13, 31) and appear to be sensitized in cardiovascular disease conditions (6, 13, 14). In models of chronic pain, sensitivity changes of MA piezo channels contribute to the development of mechanical allodynia and hyperalgesia. However, gating mechanisms responsible for setting the sensitivity of piezo channels are complex and likely involved the contribution of both biophysical and biochemical factors. Dubin et. al. showed that MA piezo currents could be augmented by inflammatory mediators such as bradykinin receptor stimulation and piezo channel sensitization results in mechanical hyperalgesia through a protein kinase C (PKC)-dependent pathway (11). Eijkelkamp et. al. found that elevated signaling through Epac1 and increases in intracellular calcium sensitize MA piezo channels (12). These data provide evidence that the biochemical aspects of sensory neurons contribute to augmenting the sensitivity of MA piezo channels. Until the data presented in this investigation, our recent work has focused on the biochemical modulation of MA piezo channels associated with exaggerated mechanoreflex. In ligated rats, we previously reported that G_q-coupled thromboxane A_2 receptors, and further downstream signaling target IP_3 receptors, within sensory neurons contribute to mechanoreflex sensitization in ligated rats. These data support a role for biochemical alterations via augmented intracellular signaling playing a role in MA channel sensitization. Interestingly, biophysical alterations in the structure of the cell (*i.e.*, cytoskeletal alterations) can impact coordinated intracellular signaling. The cytoskeleton is additionally responsible for organization and coordination of intracellular second messenger signaling. For example, A-kinase anchoring proteins are scaffolding protein that regulates signal transduction by physically bringing effectors and substrates into close proximity. Disruption of this structural protein significantly alters

coordinated intracellular signaling (4, 9, 16) and the correct localization and function of G-coupled protein receptors (1). While actin polymerization inhibition in our studies likely disrupted physical tethers of MA piezo channels and reduced static plasma membrane tension resulting in an overall reduction in mechanical sensitivity, the disruption of the cytoskeleton could have also uncoordinated intracellular signaling that would have otherwise caused biochemical sensitization of MA channels.

Several experimental considerations warrant discussion. First, atherosclerosis develops with a slow and gradual narrowing of the arteries in PAD patients whereas the rat model of simulated PAD relies on instantaneous and complete femoral artery occlusion. Nevertheless, femoral artery ligation followed by 72 hours of recovery replicates the limb blood flow patterns and exaggerated exercise pressor reflex found in PAD patients (40, 44). Second, mechanoreflex activation of passive hindlimb skeletal muscle stretch provides a lengthening stimulus to MA channels, whereas during contraction MA channels experience a shortening stimulus. However, a large majority of afferents that respond to passive stretch are also activated by contraction in rats (37). Third, actin filaments are one of three major components of the cytoskeleton. Further work should be done to more completely define the role of other cytoskeletal components in mechanoreflex activation (8). Fourth, the plasma membrane and cytoskeleton are interconnected such that both likely play a role in the gating mechanics. For example, integral membrane protein, stomatin, may form part of mechanotransduction complex that links MA channels embedded in the plasma membrane to the cytoskeleton and regulates the sensitivity of MA channels (24). Additional work needs to be done explore the contribution of plasma membrane components in the activation and modulation of MA channels.

In summary, we investigated a role of actin polymerization on chronic mechanoreflex sensitization in a rat model of simulated PAD. We found that actin polymerization inhibition with CD reduces the exaggerated mechanoreflex and exercise pressor reflex in ligated rats. These results provide evidence that MA channel sensitivity is, in part, modulated by the cytoskeleton. This investigation reveals a novel approach to our understanding of the biophysical aspects of MA channel activation and exaggerated increases in sympathetic nervous system activity and blood pressure during exercise in this patient population.

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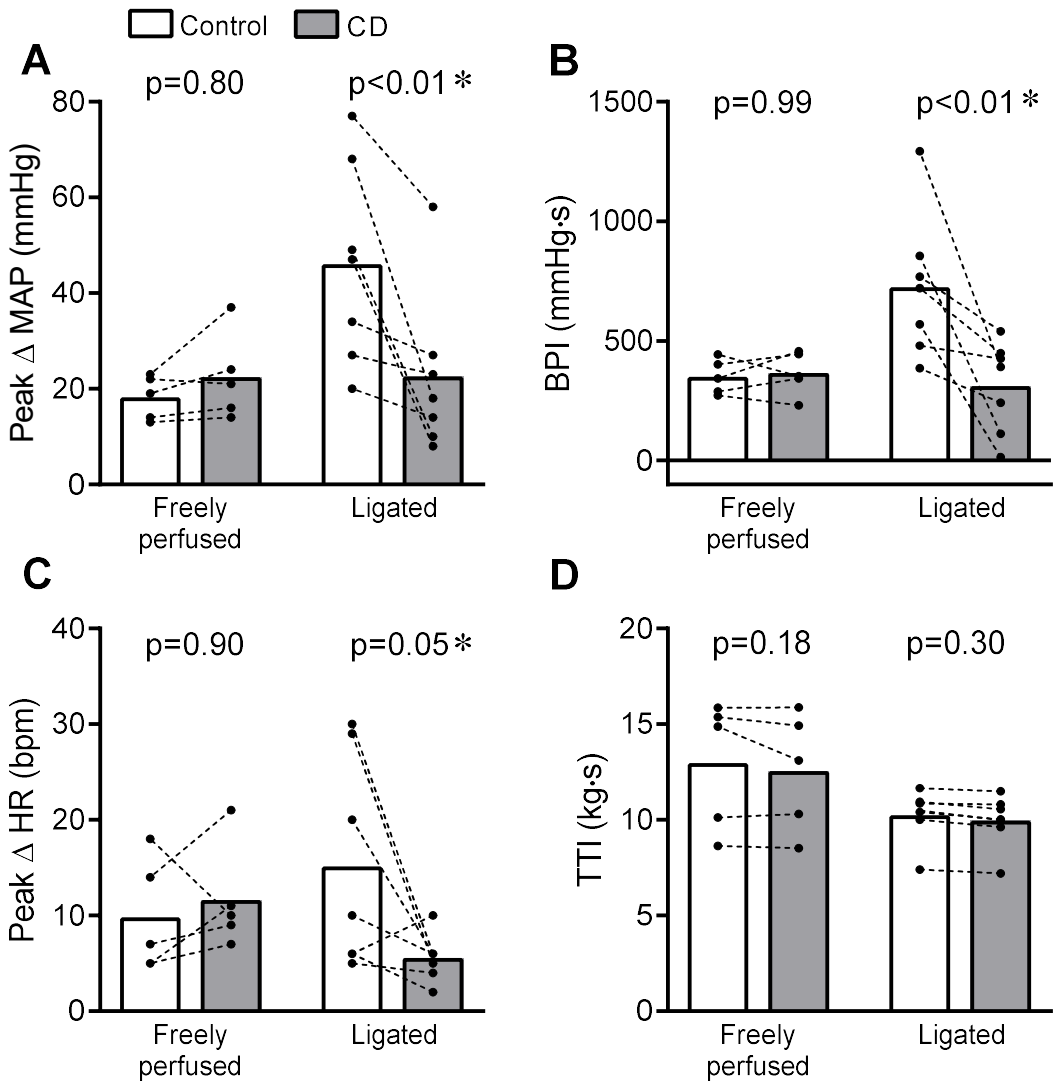
Table 4.1 ***Baseline mean arterial pressure (MAP) and heart rate (HR)***

Baseline MAP (mmHg)			
Experimental group	Control	Post condition	p-value
Freely perfused stretch (CD, n=5)	97±7	84±4	0.21
Ligated stretch (CD, n=7)	109±5	112±7	0.61
Ligated stretch (CD i.v., n=4)	124±8	114±10	0.09
Ligated stretch (0.025% DMSO, n=3)	131±4	121±10	0.35
Ligated lactic acid inj. (CD, n=4)	116±10	118±4	0.73
Ligated ATP inj (CD, n=4)	103±12	115±13	0.33
Ligated stretch (CD+10 µg phalloidin, n=5)	116±7	107±6	0.07
Ligated stretch (CD+20 µg phalloidin, n=4)	117±8	112±21	0.73
Ligated contraction (CD, n=5)	111±9	109±12	0.83
Baseline HR (bpm)			
Experimental group	Control	Post condition	p-value
Freely perfused stretch (CD, n=5)	486±20	481±15	0.63
Ligated stretch (CD, n=7)	510±9	525±20	0.49
Ligated stretch (CD i.v., n=4)	493±13	496±15	0.74
Ligated stretch (0.025% DMSO, n=3)	505±9	502±20	0.82
Ligated lactic acid inj. (CD, n=5)	511±16	511±17	0.97
Ligated ATP inj (CD, n=4)	461±16	477±11	0.34
Ligated stretch (CD+10 µg phalloidin, n=5)	491±12	497±14	0.26
Ligated stretch (CD+20 µg phalloidin, n=4)	502±20	489±19	0.40
Ligated contraction (CD, n=5)	435±33	437±28	0.88

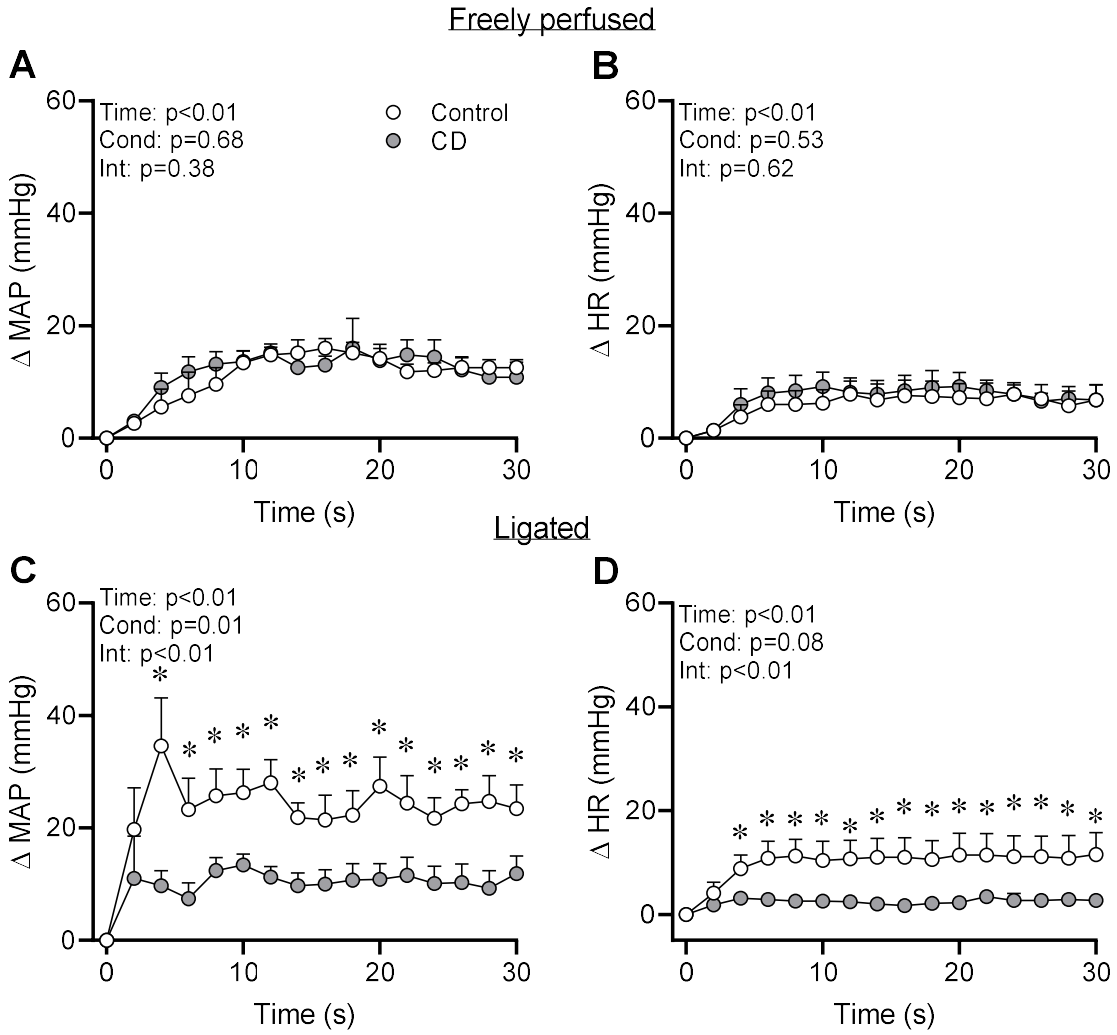
The experimental group is identified by the presence of a ligated or patent (freely perfused) femoral artery, the experimental maneuver, and the injectate. Values are mean±SEM. CD, cytochalasin D. Data were analyzed with paired Student's t-tests. Asterisks indicate statistical significance ($p \leq 0.05$).

Figure 4.1 ***Effect of actin polymerization inhibition with cytochalasin D (CD) on isolated mechanoreflex activation.***



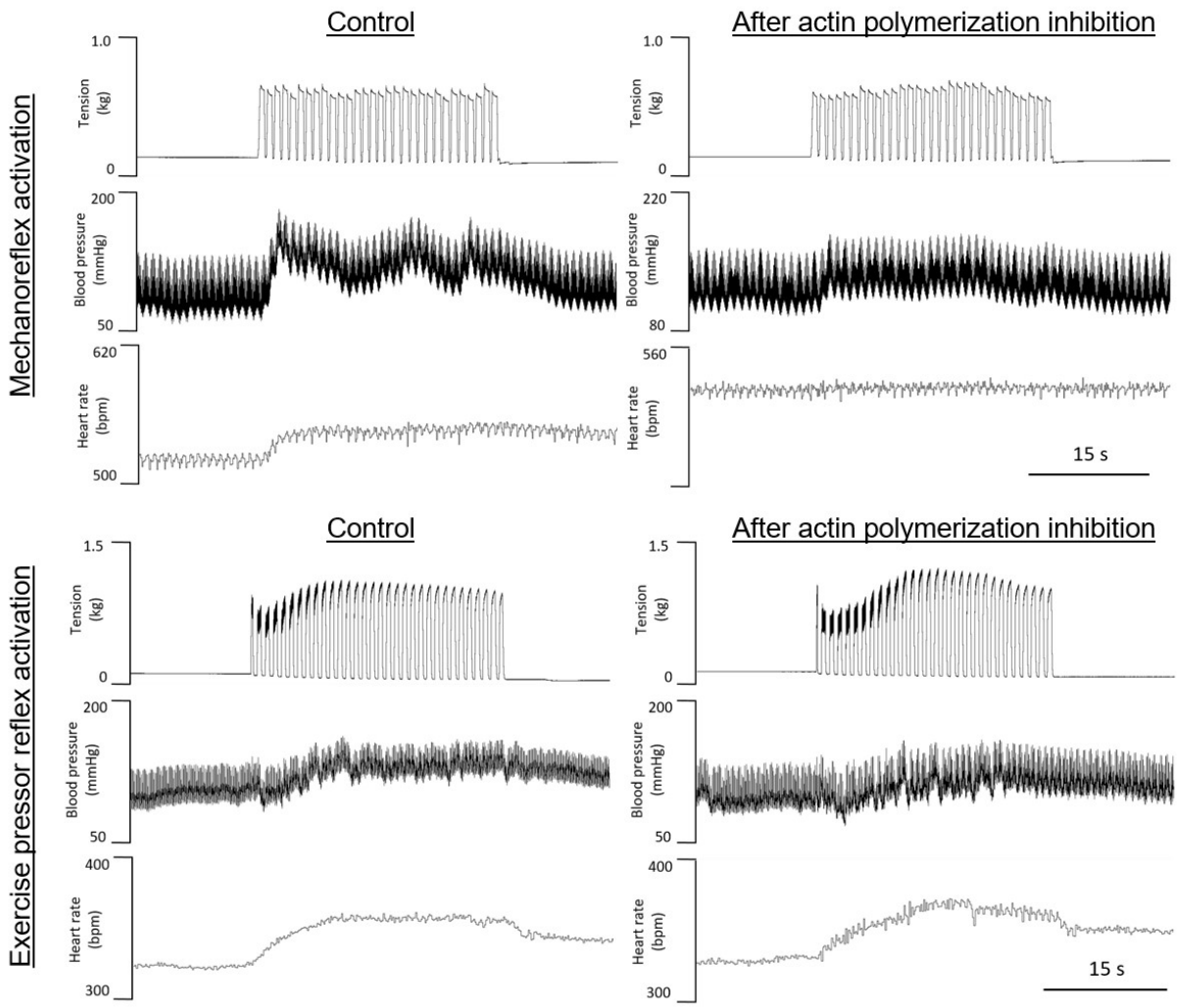
The peak Δ mean arterial pressure (peak Δ MAP, A), blood pressure index (BPI, B), and peak Δ heart rate (peak Δ HR, C) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle stretch before and after injection of CD (400 ng) into the arterial supply of the hindlimb of freely perfused (n=4) and ligated (n=7) rats. The tension-time index (Δ TTI, D) was not different between conditions for either group. Data were analyzed with Sidak multiple comparisons tests. Bars represent group mean. Asterisks indicate statistically significant ($p \leq 0.05$) differences between conditions.

Figure 4.2 ***Effect of actin polymerization inhibition with cytochalasin D (CD) on the time course of mechanoreflex activation.***



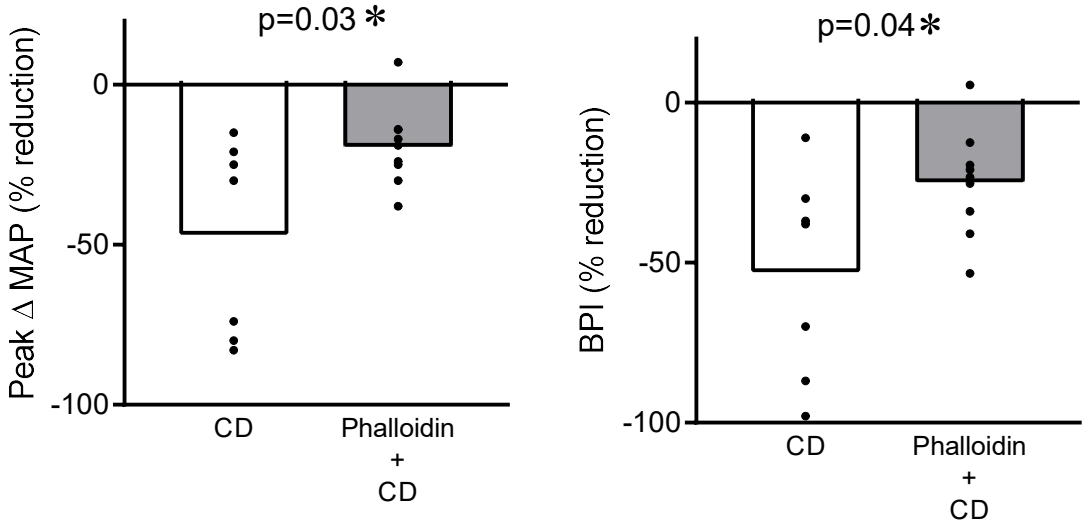
The Δ mean arterial pressure (Δ MAP, A and C) and Δ heart rate (Δ HR, B and D) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle stretch before and after injection of CD (400 ng) into the arterial supply of the hindlimb of freely perfused (top panels, n=4) and ligated (bottom panels, n=7) rats. Data were analyzed with two-way repeated measures ANOVA and Sidak multiple comparisons tests. Asterisks indicate time points where statistically significant ($p \leq 0.05$) differences exist between conditions. Cond. = condition effect, Int. = interaction.

Figure 4.3 ***Original data tracings of dynamic stretch and contraction before and after actin polymerization inhibition with cytochalasin D***



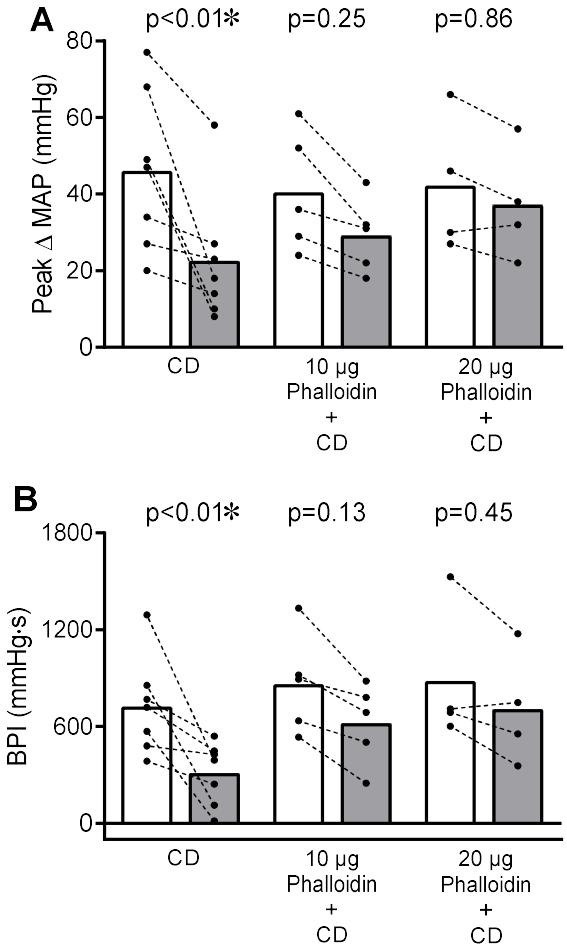
Original tracings in ligated rats of the blood pressure and heart rate response to 30 s of 1 Hz dynamic hindlimb skeletal muscle stretch (top panels) and contraction (bottom panels) before (*left*) and after (*right*) injection of actin polymerization inhibitor cytochalasin D (400 ng) into the arterial supply of the hindlimb.

Figure 4.4 ***Actin stabilization with phalloidin mitigated the effect of cytochalasin D (CD) on pressor response during isolated mechanoreflex activation.***



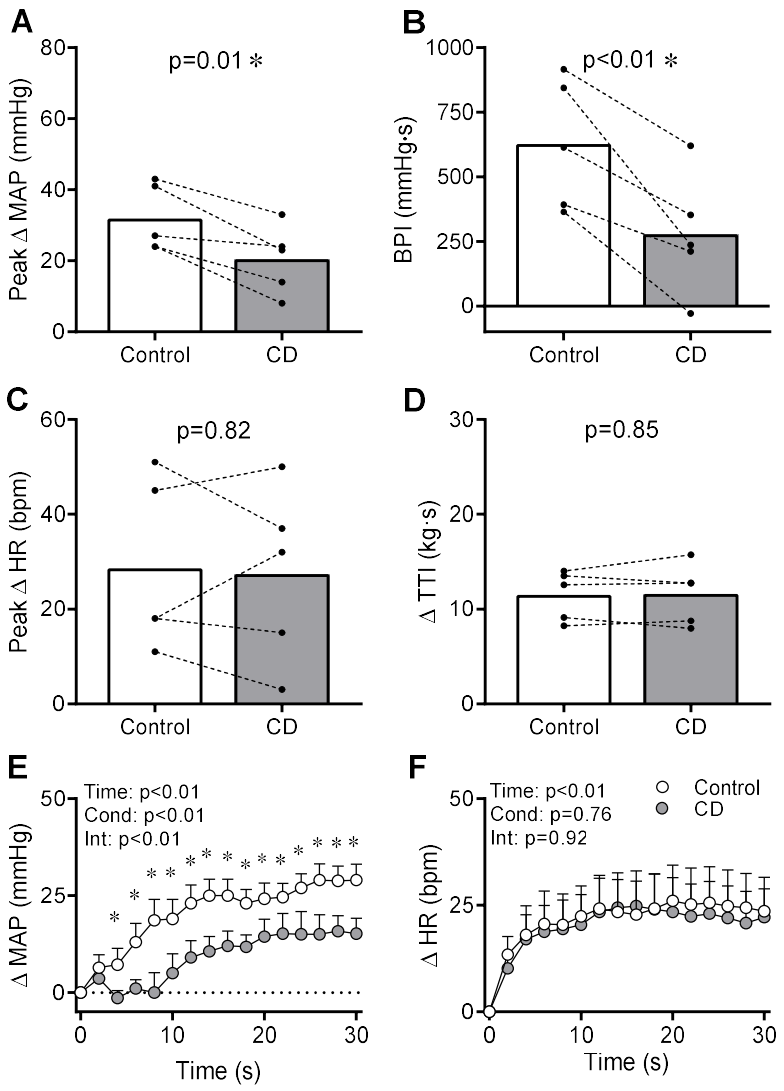
The percent reduction in the peak Δ mean arterial pressure (peak Δ MAP) and the percent reduction in the blood pressure index (BPI) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle stretch before and after injection of CD (400 ng) into the arterial supply of the hindlimb of ligated rats. "CD" = ligated rats (n=7) reported in Fig. 1 in which only CD and not phalloidin was injected. "Phalloidin + CD" = ligated rats (n=9) in which 10 µg or 20 µg of phalloidin was injected 1 hr prior to the control stretch and CD injection. The tension-time index (see *Results*) was not different between conditions for any group. Data were analyzed with Student's t-tests. Bars represent group mean. Asterisks indicate statistically significant ($p \leq 0.05$) difference between conditions.

Figure 4.5 ***The effect of phalloidin administered prior to cytochalasin D (CD) on isolated mechanoreflex activation.***



The peak Δ mean arterial pressure (peak Δ MAP, A) and blood pressure index (BPI, B) response to 30 s of 1 Hz dynamic hindlimb skeletal muscle stretch before and after injection of CD (400 ng) into the arterial supply of the hindlimb of ligated rats. "CD" = ligated rats (n=7) reported in Fig. 1 in which only CD and not phalloidin was injected. "10 µg phalloidin + CD" = ligated rats (n=5) in which 10 µg of phalloidin was injected 1 hr prior to the control stretch and CD injection. "20 µg phalloidin + CD" = ligated rats (n=4) in which 20 µg phalloidin was injected 1 hr prior to the control stretch and CD injection. The tension-time index (see Results) was not different between conditions for any group. Data were analyzed with Sidak multiple comparisons tests. Bars represent group mean. Asterisks indicate statistically significant ($p \leq 0.05$) difference between conditions.

Figure 4.6 ***Effect of actin polymerization inhibition with cytochalasin D (CD) on the exercise pressor reflex.***



The peak Δ mean arterial pressure (Δ MAP, A), blood pressure index (BPI, B), peak Δ heart rate (ΔHR, C), and MAP and HR time course (E and F) response to 30 s 1 Hz dynamic hindlimb skeletal muscle stretch before and after injection of CD (400 ng) into the arterial supply of the hindlimb of ligated rats (n=5). The tension-time index (Δ TTI, D) was not different between conditions Data are analyzed with paired Student's t-tests (A-D) or two-way repeated measures ANOVA with Sidak multiple comparisons tests (E and F). Bars are group mean. Asterisks indicate statistically significant ($p \leq 0.05$) differences between conditions. Cond. = condition effect, Int. = interaction.

Chapter 5 - Conclusion

The overall aim of this sequence of studies presented as part of my dissertation was to determine biochemical and biophysical mechanistic causes of MA channels sensitization which contributes to the exaggerated mechanoreflex and exercise pressor reflex in a rat model of simulated peripheral artery disease (PAD). In chapter 2, we found that blockade of TxA_2 receptors reduced the exaggerated pressor and cardioaccelerator response to dynamic hindlimb skeletal muscle stretch and contraction in a rat model of simulated PAD. In chapter 3, we also found that IP_3 receptor blockade, a component of the second messenger signaling linked to G_q protein coupled receptors such as TxA_2 receptors, reduced the exaggerated pressor and cardioaccelerator response to dynamic hindlimb skeletal muscle stretch and contraction in a rat model of simulated PAD. These data suggest that TxA_2 receptors contribute to the exaggerated mechanoreflex, in part, through their second messenger signaling pathway resulting in IP_3 production. In chapter 4, we switched focus to biophysical aspects of MA channel regulation. We found that actin polymerization inhibition reduced exaggerated pressor and cardioaccelerator response to dynamic hindlimb skeletal muscle stretch and contraction in a rat model of simulated PAD. Together, these data provide preclinical evidence of cellular signaling and structural contributors to MA channel sensitivity that play a role in the exaggerated mechanoreflex in PAD patients. PAD patients experience exaggerated increases in blood pressure during exercise and reduced exercise tolerance. This investigation reveals important mechanisms within thin fiber sensory neurons that may contribute to reflex-mediated sympathoexcitation and exaggerated blood pressure increases during exercise in this patient population.

Appendix A - Curriculum Vitae

KORYNNE ROLLINS

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EDUCATION

Kansas State University

Ph.D. (Physiology) 2018-Present

Dissertation: "Mechanistic bases of the exaggerated mechanoreflex in peripheral arterial disease"

Kansas State University

M.S. (Kinesiology) May 2018

Thesis: "Bradykinin does not acutely sensitize the reflex pressor response during hindlimb skeletal muscle stretch in decerebrate rats"

Kansas State University

B.S. (Kinesiology) December 2015

Minor: Spanish

AWARDS

Cum Laude December 2015

Masters Graduate Student of the Year, Kansas State Department of Kinesiology May 2017

American Kinesiology Association Masters Scholar May 2018

Caroline tum Suden/Hellebrandt Professional Opportunity Award (APS) April 2020

Graduate Teaching Assistant of the Year, Kansas State Department of Kinesiology May 2020

GRANTS

F31 National Institutes of Health Predoctoral Fellowship (FOA: PA-19-195) December 2019

Title: Mechanistic bases for the exaggerated mechanoreflex in peripheral arterial disease

Score: Impact: 37, Percentile: 30

Status: Awarded – August 2020

TEACHING EXPERIENCE

Kansas State University

Graduate Teaching Assistant August 2016 – May 2020

- Instruct five undergraduate Kinesiology laboratory classes including Biobehavioral Bases of Physical Activity, Research Methods, Exercise Physiology, Exercise Testing and Prescription, and Anatomy and Physiology
- Grade examinations, evaluate laboratory reports, and record grades
- Assist in the revision and creation of laboratory course material

Graduate Research Assistant August 2016 - Present

- Conduct autonomic neurophysiology rodent experiments
- Conduct research, analyze data, and write scientific manuscripts
- Train incoming students on surgical techniques, laboratory equipment and software

Undergraduate physiology laboratory coordinator August 2019 – May 2020

- Procure materials and coordinate equipment usage for undergraduate physiology laboratories
- Communicate with other graduate teaching assistants, students, and faculty to ensure proper execution of protocols
- Monitor, arrange, and delegate responsibilities to other teaching assistants

ADDITIONAL HIGHER EDUCATION EXPERIENCE

K-State First Learning Assistant August 2014 – 2016

- Mentored a group of incoming freshmen and planned co-curricular events for group bonding
- Prepared and led group discussions on topics related to the field of Kinesiology and career of Physical Therapy

K-State Connect Supplemental Instructor for the Department of Kinesiology August 2014 – 2016

- Planned and organized tutoring sessions for the class Biobehavioral Bases of Physical Activity covering topics including physiology and behavioral studies

K-State Recreational Services ACE Certified Personal Trainer August 2014 – 2016

- Designed weekly workout plans for clients and trained clients in proper lifting techniques and use of equipment
- CPR/First Aid certified

K-State CrossFit CrossFit Coach May 2016 – January 2021

- Provide coaching and training in the methodologies of CrossFit to ensure safety and enable performance toward planned goals of patrons

PUBLICATIONS AND PAPERS

Rollins, K. S., Smith, J. R., Esau, P. J., Kempf, E. A., Hopkins, T. D., & Copp, S. W. (2017). Bradykinin does not acutely sensitize the reflex pressor response during hindlimb skeletal muscle stretch in decerebrate rats. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. doi:10.1152/ajpregu.00187.2017

Kempf EA, Rollins KS, Hopkins TD, Butenas AL, Santin JM, Smith JR, Copp SW. (2017) Chronic femoral artery ligation exaggerates the pressor and sympathetic nerve responses during dynamic skeletal muscle stretch in decerebrate rats. American Journal of Physiology – Heart and Circulatory. doi: 10.1152/ajpheart.00498.2017

Esau, P. J., Gittemeier, E. M., Opoku-Acheampong, A. B., Rollins, K. S., Baumfalk, D. R., Poole, D. C., Musch T. I., Behnke B. J., Copp, S. W. (2017) Prostate cancer reduces endurance exercise capacity in association with reductions in cardiac and hindlimb skeletal muscle mass in the rat. American Journal of Cancer Research. 1;7(12):2566-2576. eCollection 2017

Sanderson, B. C., Rollins, K. S., Hopkins, T. D., Butenas, A. L., Felice, K. P., Ade, C. J., & Copp, S. W. (2019). GsMTx4 reduces the reflex pressor response during dynamic hindlimb skeletal muscle stretch in decerebrate rats. Physiological reports. doi: 10.14814/phy2.13974

Rollins, K. S., Hopkins, T. D., Butenas, A. L., Felice, K. P., Ade, C. J., & Copp, S. W. (2019). Cyclooxygenase inhibition does not impact the pressor response during static or dynamic mechanoreflex activation in healthy decerebrate rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. doi: 10.1152/ajpregu.00080

Butenas, A. L., Hopkins, T. D., Rollins, K. S., Felice, K. P., & Copp, S. W. (2019). Investigation of the mechanisms of cyclooxygenase-mediated mechanoreflex sensitization in a rat model of simulated peripheral artery disease. American Journal of Physiology-Heart and Circulatory Physiology, 317(5), H1050-H1061.

Rollins, K. S., Butenas, A. L., Felice, K. P., Matney, J. E., Williams, A. C., Kleweno, T. E., & Copp, S. W. (2020). Thromboxane A2 Receptors Mediate Chronic Mechanoreflex Sensitization in a Rat Model of Simulated Peripheral Artery Disease. American Journal of Physiology-Heart and Circulatory Physiology.

Butenas, A. L., Rollins, K. S., Matney, J. E., Williams, A. C., Kleweno, T. E., Parr, S. K., Hammond S. T., Ade C. J., Hageman K. S., Musch T. I., Copp, S. W. (2020). No effect of endoperoxide 4 or thromboxane A2 receptor blockade on static mechanoreflex activation in rats with heart failure. Experimental Physiology, 105(11), 1840-1854.

Butenas, A. L., Rollins, K. S., Williams, A. C., Parr, S. K., Hammond, S. T., Ade, C. J., ... & Copp, S. W. (2021). Exaggerated sympathetic and cardiovascular responses to dynamic mechanoreflex activation in rats with heart failure: Role of endoperoxide 4 and thromboxane A2 receptors. Autonomic Neuroscience, 232, 102784.

PRESENTATIONS

Rollins, K. S., Smith, J. R., Esau, P. J., Kempf, E. A., Hopkins, T. D., & Copp, S. W. (2017). Bradykinin does not acutely sensitize the reflex pressor response during hindlimb skeletal muscle stretch in decerebrate rats. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. doi:10.1152/ajpregu.00187.2017

Abstract presented at Experimental Biology, Chicago, IL

Esau, P. J., Gittemeier, E. M., Opoku-Acheampong, A. B., Rollins, K. S., Baumfalk, D. R., Poole, D. C., Musch T. I., Behnke B. J., Copp, S. W. (2017) Prostate cancer reduces endurance exercise capacity in association with reductions in cardiac and hindlimb skeletal muscle mass in the rat. American Journal of Cancer Research. 7(12): 2566–2576

Abstract presented at Experimental Biology, Chicago, IL

Rollins, K. S., Butenas, A. L., Kempf, E. A., Hopkins, T. D., Sanderson, B. C., & Copp, S. W. (2018). Blood pressure responses to hindlimb arterial bradykinin injection are mediated by bradykinin 2 receptors in decerebrate rats. The FASEB Journal, 32, 725-6.

Abstract presented at Experimental Biology, San Diego, CA

Rollins, K. S., Hopkins, T. D., Butenas, A. L., Felice, K. P., Ade, C. J., & Copp, S. W. (2019). Cyclooxygenase inhibition does not impact the pressor response during static or dynamic mechanoreflex activation in healthy decerebrate rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. doi: 10.1152/ajpregu.00080

Abstract presented at Experimental Biology Orlando, FL

Rollins, K. S, Butenas, A. LE., Williams, A. C., & Copp, S. W. TRPV1 channel stimulation reduces the mechanoreflex in decerebrate rats. *The FASEB Journal*, *35*.

Abstract presented at Experimental Biology, virtually 2021

LANGUAGES

English– native language

Spanish–speak, read, and write with basic competence

MEMBERSHIPS

American Physiological Society