# Role of bradykinin receptor B2 in mechanoreflex activation in a rat model of simulated peripheral artery disease

by

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# Abstract

The exercise pressor reflex, and the mechanical component of the exercise pressor reflex specifically (i.e., 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 ligated ~24-72 hours before the experiment. In the rat, the mechanisms and outcomes of mechanoreflex activation may be investigated by rhythmically stretching the hindlimb skeletal muscles to mimic the pattern of mechanical stimulation present during locomotion. The purpose of the present study was to investigate the role played by bradykinin B2 receptors (B2-Rs) on the sensory endings of thin fiber muscle afferents in the chronic sensitization of the mechanoreflex in the rat ligated femoral artery model of simulated PAD. We hypothesized that in decerebrate, unanesthetized rats, B2-R blockade with 100 ng of the B2-R antagonist HOE-140 would result in a greater attenuation of the pressor and cardioaccelerator response to 30 seconds of 1 Hz dynamic hindlimb skeletal muscle stretch in rats with a femoral artery ligated for ~72 hours compared to rats subjected to a sham procedure in which the femoral artery remained freely perfused. In the ligated condition (n = 14), we found that HOE-140 had no effect on the peak ΔMAP (pre: 37 ± 4, post: 32 ± 5 mmHg, p = 0.14) or peak ΔHR (pre: 15 ± 3, post: 15 ± 2 bpm, p = 0.78) response to stretch. In the freely perfused condition (n = 5), we likewise found that HOE-140 had no effect on the peak ΔMAP (pre: 16 ± 5, post: 16 ± 3 mmHg, p = 0.79) or peak ΔHR (pre: 7 ± 3, post: 8 ± 2 bpm, p = 0.91) response to stretch. Based on these results, we conclude that B2-R signaling in sensory neurons is not required to produce the chronic mechanoreflex sensitization present in the rat model of simulated PAD in which a femoral artery is chronically ligated.

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# Acknowledgements

I would like to thank Dr. Steven Copp for all the opportunities and guidance he has given me, as well as Korynne Rollins and Alec Butenas for welcoming me and giving me every chance to learn. I would also like to thank my family, particularly my mother, Dr. Andria Giesen, for her unwavering endurance and my sisters, Aspen Williams and Alex Fender, for all the tea we have shared.

# Dedication

For Dr. Marlan “Doc” Francis, who would have loved to be here.

# Chapter 1 - Introduction

The exercise pressor reflex (EPR) is the phenomenon wherein skeletal muscle afferent stimulation produced by skeletal muscle contraction results in increased sympathetic nerve activity (SNA) and mean arterial pressure (MAP; Mitchell, Kaufman & Iwamoto, 1983). These afferents innervating contracting skeletal muscles are comprised of small, thinly myelinated group III and fine, unmyelinated group IV fibers (McCloskey & Mitchell 1972). The reflex arc stemming from the metabolic stimulation of these afferents is known as the metaboreflex, and that of the mechanical stimulation is referred to as the mechanoreflex. Skeletal muscle contraction during physical activity results in conformational changes in the sensory endings of these afferents thereby activating mechanically-sensitive channels (Copp et al., 2016), particularly among group III afferents which are more mechanically sensitive (Hayes et al., 2008; McCord & Kaufman 2010; Mense & Stahnke, 1983; Mitchell, Kaufman & Iwamoto, 1983). In patients with peripheral artery disease (PAD), constituting approximately 200 million people in the world (Shu & Santulli, 2018), these sensory neurons develop increased responsiveness to muscle contraction, resulting in EPR exaggeration (Baccelli et al., 1999; Bakke et al., 2007; Muller et al., 2012). This exaggerated blood pressure response to exercise is an important predictor of mortality (de Liefde et al., 2008).

A cyclooxygenase (COX) metabolite-mediated pressor response exaggeration has been demonstrated in PAD patients during low-intensity plantar flexion, a form of relatively isolated mechanoreflex activation in humans (Muller et al., 2015). A COX-mediated pressor response exaggeration has also been demonstrated in a rat model of simulated PAD in which a femoral artery is chronically (~72 hours) ligated during isolated mechanoreflex activation produced by rhythmically stretching the hindlimb skeletal muscles (Butenas et al., 2019). This hindlimb

muscle stretch model of isolated mechanoreflex activation allows investigators to examine the cellular mechanisms of mechanoreflex exaggeration independent from the production of contraction-induced metabolic stimuli. Using the combination of the ligated femoral artery simulated PAD model and the isolated mechanoreflex activation model, we found recently that thromboxane $A_2$ receptors ($TxA_2$-Rs), the principal receptor for the COX metabolite thromboxane $A_2$, contribute importantly to the chronic mechanoreflex sensitization (Rollins et al., 2020). $TxA_2$-Rs on sensory neurons are coupled to $G_q$ proteins, a trait that is shared by the constitutively expressed bradykinin B2 receptors (B2-Rs; Marceau et al., 2020).

In healthy conditions, the actions of the autacoid bradykinin (BK) are well characterized. Static skeletal muscle contraction releases BK (Stebbins et al. 1990), and exogenous BK has been shown to stimulate both group III and IV afferents, with greater responsiveness observed in group III afferents (Mense, 1977). The reduction of BK availability (Stebbins & Longhurst 1986), as well as the inhibition of B2-R (Pan, Stebbins & Longhurst, 1993) decreased the pressor response to static skeletal muscle contraction in the cat. Specifically, Leal et al. (2013) found, in healthy cats, that B2-R inhibition with HOE-140 attenuated the response of group III afferents to electrically-induced static skeletal muscle contraction. In the rat model of simulated PAD, Lu et al. (2013) showed a greater B2-R protein expression in the dorsal root ganglia (DRG) associated with the afferents innervating rat hindlimbs subjected to femoral artery ligation, and furthermore were able to demonstrate a greater attenuation of the pressor response to static hindlimb skeletal muscle stretch with B2-R inhibition in rats with ligated hindlimbs compared with that of freely perfused. Particularly relevant to the rat simulated PAD model, Stebbins et al. (1990) showed in cats that BK synthesis measured in the hindlimb venous circulation during muscle contraction is exaggerated when the iliac artery is occluded compared to when it is freely perfused.

Collectively, the data summarized above provide indirect evidence for an augmented role of BK-B2-R signaling in the exaggerated mechanoreflex activated by dynamic hindlimb skeletal muscle stretch in the rat simulated PAD model.

Detailing the mechanisms of chronic mechanoreflex sensitization in PAD is a critical step in the development of novel therapeutic agents designed to improve blood pressure control in this patient population. Therefore, the purpose of the present study was to investigate the role of B2-R activity in chronic mechanoreflex sensitization found in the rat model of simulated PAD. We tested the hypothesis that injection of 100 ng of the B2-R antagonist HOE-140 into the arterial supply of the hindlimb would reduce the pressor response to 30 seconds of 1 Hz dynamic hindlimb skeletal muscle stretch in rats with freely perfused hindlimbs. Moreover, we tested the hypothesis that B2-R blockade in rats subjected to a femoral artery ligation surgery ~72 hours before the experiment would reduce the pressor response to dynamic stretch to a greater extent than the reduction found in rats with freely perfused hindlimbs.

# Chapter 2 - Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The male, ~12-15 week old Sprague-Dawley rats (Charles River Laboratories) used in the experiments (n = 34) were housed paired in cages in temperature- and light-controlled accredited facilities operating at ~22°C on a 12-hour interval light-dark cycle with standard rat chow and water available ad libitum (body weight = 391 ± 10 g). Following experimental protocols, the decerebrate (see below) rats were killed via intravenous injections of >3 mg/kg potassium chloride.

*Femoral Artery Ligation and Sham Procedures*: In 26 rats, ~72 hours prior to the terminal experimental protocol, a femoral artery ligation surgery was performed wherein each rat was anesthetized with 2% isoflurane anesthesia balanced with oxygen and the left femoral artery was surgically exposed and ligated with 5-0 silk suture ~3-5 mm distal to the inguinal ligament. These rats constitute the "ligated" group. In 8 additional rats, the same procedure was done without tying the suture, and instead only passing the suture under the artery to be removed in its entirety. These rats constitute the "freely perfused" group. In each of these rats the incision was closed, and meloxicam (1-2 mg/kg) was administered subcutaneously as an analgesic.

*In Vivo Experimental Preparation*: Prior to the experimental protocol, each rat was anesthetized with 5% isoflurane balanced with oxygen. Adequate anesthesia was confirmed by the absence of toe-pinch and blink reflexes. Body core temperature was measured with a rectal probe and maintained at ~37°C via an automated heating system (Harvard Apparatus) and heat lamp. The trachea was cannulated, and the lungs were mechanically ventilated (Harvard Apparatus) with a reduced (2% isoflurane-balance $O_2$) gaseous mixture until the decerebration

was completed (see below). The right jugular vein and both common carotid arteries were then cannulated with PE-50 catheters for the administration of fluids and drugs and for measurement of arterial blood pressure (Physiological Pressure Transducer, AD Instruments) and arterial blood gas analysis, respectively. The left superficial epigastric artery was cannulated with a PE-8 catheter. The tip of the catheter was placed near the junction of the superficial epigastric artery and the femoral artery, and a reversible snare of 2-0 silk suture was placed around the left iliac artery and vein, proximal to the position of the superficial epigastric artery catheter. The left triceps surae muscles, namely the gastrocnemius, soleus, and plantaris, were exposed by reflecting the overlaying skin and skeletal muscles. The calcaneus bone was severed, and one end of a string was tied to the distal Achilles tendon and severed calcaneus while the other end was attached to a force transducer (Grass FT03) and manually operated rack and pinion.

Rats were then secured in a Kopf stereotaxic frame. Electrocardiogram electrodes were attached to monitor heart rate, and 0.2 mg dexamethasone was administered intravenously to minimize brainstem swelling during and following decerebration. All brain tissue rostral to the superior colliculi was aspirated via vacuum pump (Sapru & Krieger, 1978). Pressure was applied within the cranial vault using gauze, and once bleeding was controlled, Kwik-Sil (World Precision Instruments) silicone adhesive was used to fill the cavity. At this point, anesthesia was terminated, and an anesthesia "washout" of $\geq$ 1 hour was completed before any experimental procedure began. In four of the ligated rats, isoflurane was reduced from 2% to 0.5% immediately after decerebration for a short period of time rather than wholly terminated. Prior to undergoing the experimental protocol (see below), this lower concentration of the anesthetic had been terminated for ~45-60 minutes. Decerebration was chosen over anesthesia as best painless experimentation practice because anesthesia is known to alter the mechanoreflex and exercise

pressor reflex in the rat (Smith et al., 2001), and specifically the gaseous anesthetic used in these experiments has been shown to decrease MAP via reductions in sympathetic outflow with regional differences in the degree of this effect (Åneman et al., 1995). Rats were paralyzed with intravenous injection of pancuronium bromide (~1 mg/kg) to prevent any spontaneous movement from confounding the intended isolated mechanical experimental stimulus. Periodically, arterial blood samples (~75 μl) were taken and pH and blood gases were analyzed. If analysis revealed pH or $PCO_2$ values outside of normal ranges (pH: 7.35-7.45; $PCO_2$: ~38-40 mmHg), sodium bicarbonate administration or ventilation adjustments were performed as necessary.

Following all experiments but prior to euthanasia, Evan's blue dye was injected into the superficial epigastric artery catheter to confirm injectate had access to the triceps surae muscle circulation. Triceps surae muscles stained blue in all experiments used in data analysis. Rats were subsequently euthanized via intravenous injection of saturated (>3 mg/kg) potassium chloride.

*Bradykinin Injection Experimental Protocol*: In a mixed (three freely perfused and four ligated) group of rats, the efficacy of the 100 ng dose of HOE-140 to block B2-Rs was tested. Baseline blood pressure and heart rate were recorded. Then, the iliac snare was tightened, and bradykinin (0.5 μg dissolved in 0.2 ml of saline) was injected as a bolus into the superficial epigastric artery catheter. The snare was released when the MAP returned to baseline values. Following recovery (~5 min) from this control injection of bradykinin, the iliac snare was again tightened, and 100 ng of HOE-140 dissolved in 0.2 ml of saline was injected into the superficial epigastric artery catheter. The snare was left taut for five minutes and subsequently relaxed for

10 minutes. Bradykinin (0.5 μg dissolved in 0.2 ml of saline) was again injected into the superficial epigastric artery catheter exactly as described for the control condition.

*Experimental Protocol Activating the Muscle Mechanoreflex*: In order to examine the pressor response to mechanoreflex activation independent of the metaboreflex, a 1 Hz dynamic muscle stretch protocol was performed on 19 rats (ligated n = 14, freely perfused n = 5). Baseline muscle tension in the triceps surae muscles was set at ~80-100 g. Following baseline data collection an investigator initiated the stretch protocol wherein the pinion was manually rotated back and forth along the rack at a 1 Hz frequency with the aid of a metronome for 30 seconds. The investigator aimed for equal distances between each rotation to produce a consistent force of tension developed (~0.7-0.9 kg) on the triceps surae muscles with each stretch. ~5 minutes after this control stretch, the iliac snare was tightened and 100 ng of HOE-140 dissolved in 0.2 ml of saline was injected into the left superficial epigastric artery catheter to be distributed in the left hindlimb circulation for 5 minutes, after which the snare was relaxed and the hindlimb was allowed to reperfuse for 10 minutes. Baseline tension was then reset, and the stretch maneuver was repeated as described above.

*Tissue Collection*: In eight ligated rats not used for in vivo experiments, the $L_4$ and $L_5$ dorsal root ganglia (DRG) were harvested from both the left, ligated hindlimb and the right, non-operated freely perfused hindlimb in euthanized rats. Samples were placed in 2 ml bead mill tubes containing ~0.5 g of 1.4 mm diameter ceramic beads and 350 μl of β-Mercaptoethanol. The samples were then homogenized for 1 minute at 5 m/s using Bead Mill 4 (FisherbrandTM). Total protein and mRNA from the samples were prepared with the Nucleospin miRNA/Protein Kit (Macherey-Nagel) in accordance with the manufacturer's instructions. Total protein and mRNA

concentrations were determined using the Quibit 2.0 Fluorometer (Life Technologies) and stored in a -80°C freezer.

*Western Blots*: Protein samples (30 μg) were separated on 4-12% Bis-Tris Protein Gels (Invitrogen™) by gel electrophoresis in MES running buffer (Invitrogen™) using 200 V for 22 minutes. Gels were then transferred to mini-PVDF membranes using the iBlot 2 Dry Transfer Device (Invitrogen™). Membranes were incubated for ~3 hours with the iBind device with iBind solution (Invitrogen™) with a primary antibody for the bradykinin B2-R diluted 1:100 (Santa Cruz Biotechnology), a primary antibody for GAPDH diluted 1:1000 (Thermofisher Scientific), along with a Goat anti-Mouse IgG (H+L), secondary antibody conjugated with Horse Radish Peroxidase diluted 1:1000 (Thermofisher Scientific). Membranes were then incubated for 5 minutes with 6 ml of SupersSignal™ West Pico PLUS Chemiluminescent Substrate (Thermofisher) and imaged with C-DiGit® Blot Scanner (Li-Cor). The protein bands were quantified and analyzed using the Image Studio software (Li-Cor).

*Quantitative Reverse Transcriptase Polymerase Chain Reaction*: Complementary DNA (cDNA) was synthesized from RNA isolates (see above) using the High Capacity RNA-cDNA™ kit (ThermoFisher Scientific) according to the manufacturer's instructions. Quantitative reverse transcriptase polymerase chain reaction was then performed on the cDNA samples using TaqMan gene expression assays specific for B2-R (Sequence proprietary; assay ID: Rn04338900_m1) and GAPDH (forward primer: 5′-ACCGCCTGTTGCGTGTTA-3′ and reverse primer: 5′-CAATCGCCAACGCCTCAA-3′; assay ID: Rn01775763_g1). All samples were run in triplicate for the gene of interest, and the endogenous control (GAPDH). The results were analyzed with the comparative threshold (ΔΔCt) method.

*Data Analysis*: Muscle tension, blood pressure, and heart rate (HR) were measured and recorded in real time with a PowerLab and LabChart data acquisition system (AD Instruments). Baseline mean arterial pressure (MAP) and HR were determined from the baseline periods that preceded each maneuver. For the stretch maneuvers, the peak increases in MAP (peak Δ MAP) and HR (peak Δ HR) were calculated as the difference between the peak values that occurred during the maneuvers and their corresponding baseline values. For the bradykinin injection experiments, the peak Δ MAP and peak Δ HR were calculated as the differences between the peak values that occurred within 30 seconds of completion of the injection and their corresponding baseline values. The tension-time indices (TTIs) and blood pressure indices (BPIs) were calculated by integrating the area under the data signal during the stretch maneuver and subtracting the integrated area under the data signal during the baseline period. Data were first assessed for the presence of a normal distribution, equal variance, and/or effectiveness of pairing as appropriate. Data were then analyzed with paired or unpaired student's t-tests or two-way ANOVA and Holm-Šidák multiple comparisons tests as appropriate. A Cohen's d test was used to calculate effect size as appropriate. Data are represented as Mean ± Standard Error of Mean. Statistical significance was defined as $p \leq 0.05$.

# Chapter 3 - Results

*Effect of HOE-140 Administration on the Pressor Response to Bradykinin Injection:* Summarized values for the bradykinin injection experiments are shown in **Figure 1** (n = 7). The injection of BK into the arterial supply of the hindlimb produced a rapid and distinct increase in MAP that was significantly reduced following HOE-140 administration. BK injection also produced a small but consistent cardioaccelerator response which was unaffected by HOE-140 administration. The significant reduction of the pressor response to BK injection following HOE-140 indicated that the 100 ng dose of HOE-140 effectively blocked B2-Rs. Baseline MAP (pre: 113 ± 10, post: 110 ± 9 mmHg, p = 0.51) and HR (pre: 523 ± 20, post: 523 ± 23 bpm, p = 0.89) were not different between pre- and post-HOE-140 conditions in these BK injection experiments.

*Effect of Femoral Artery Ligation on Mechanoreflex Activation:* The effects of femoral artery ligation on the mechanoreflex are shown in **Figure 2.** The peak ΔMAP and BPI response to tendon stretch were higher in the ligated group (n = 14) compared with the freely perfused group (n = 5, $P \leq 0.05$). The peak ΔHR response trended toward being higher in the ligated group (p = 0.06; Cohen's d = 0.89). Neither baseline MAP (p = 0.50) nor HR (p = 0.70) were different between groups (see "pre" data in next paragraph).

*Effect of B2-R Inhibition on Mechanoreflex Activation:* In both the ligated and freely perfused groups, HOE-140 had no significant effect on peak ΔMAP, BPI, and peak ΔHR response to stretch (**Figure 2**). **Figure 3** shows the time courses of the ΔMAP and ΔHR responses. There was no significant main effect of HOE-140 (condition) or interaction effect in either group. HOE-140 did, however, significantly reduce ΔHR at several time points in the ligated group. In the freely perfused group, there was no difference in baseline MAP (pre: 102 ± 11, post: 94 ± 9 mmHg; p = 0.51) or HR (pre: 520 ± 16, post: 505 ± 12 bpm, p = 0.39) between

conditions. Likewise, in the ligated group baseline MAP (pre: 109 ± 4, pre: 114 ± 4 mmHg, p = 0.32) and HR (pre: 514±8, post: 517±7 bpm, p = 0.61) were not different between conditions.

*Western Blot and Quantitative Reverse Transcriptase Polymerase Chain Reaction:* There was no significant difference in B2-R protein or mRNA expression between $L_4$ and $L_5$ DRG tissue coinciding with the innervation of the ligated limb and $L_4$ and $L_5$ DRG tissue coinciding with the innervation of the freely perfused limb (**Figure 4**).

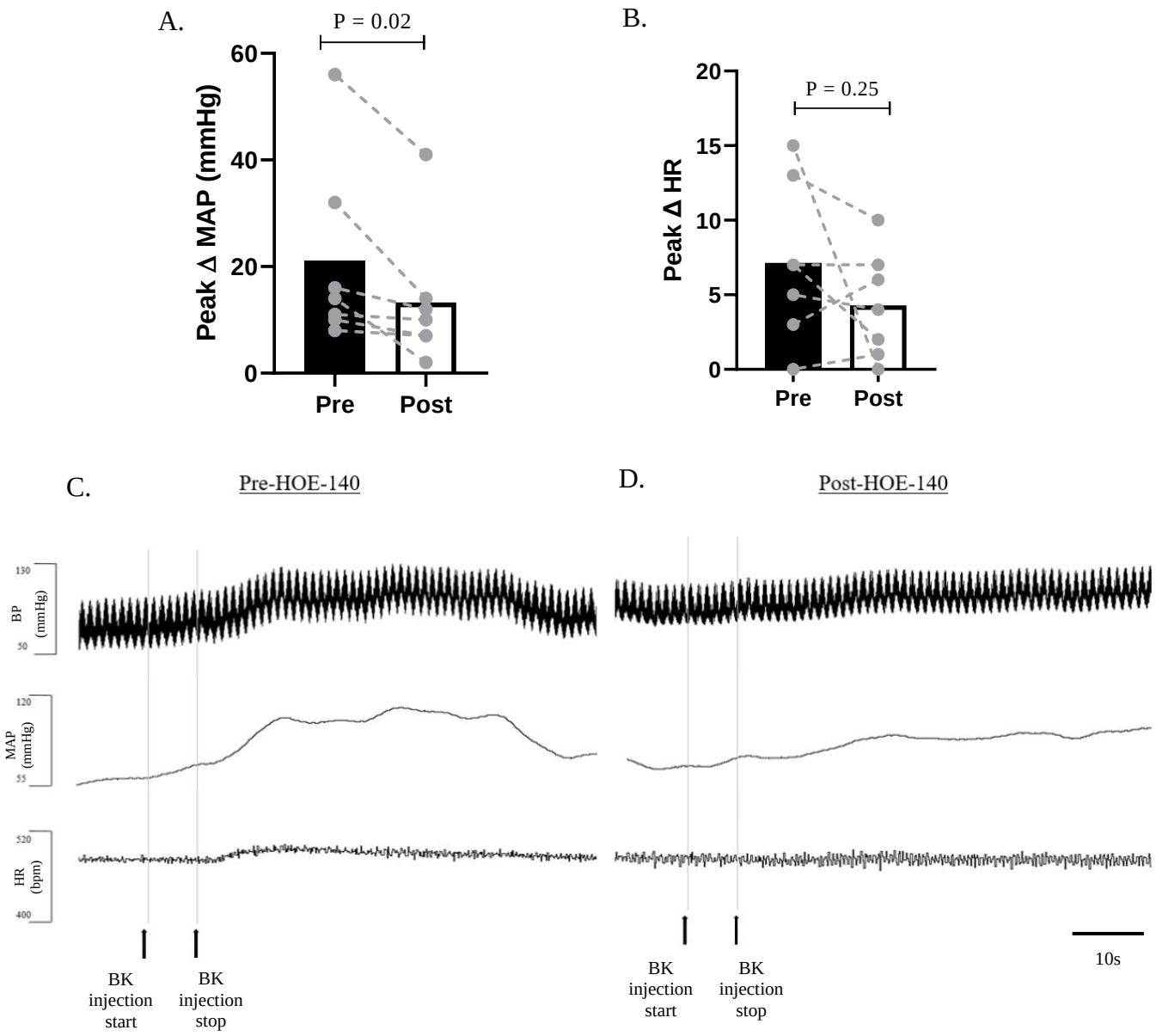


**Figure 1: Cardiovascular response to BK injection pre- and post-HOE-140 administration**

The peak ΔMAP (A) but not peak ΔHR (B) response to BK injection was significantly reduced after HOE-140 administration. Panels C and D are original tracings of the BP, MAP, and HR response to BK injection before (C) and after (D) HOE-140 administration. Arrows indicate when BK injection started and stopped. Data were compared with paired Student's t-tests.

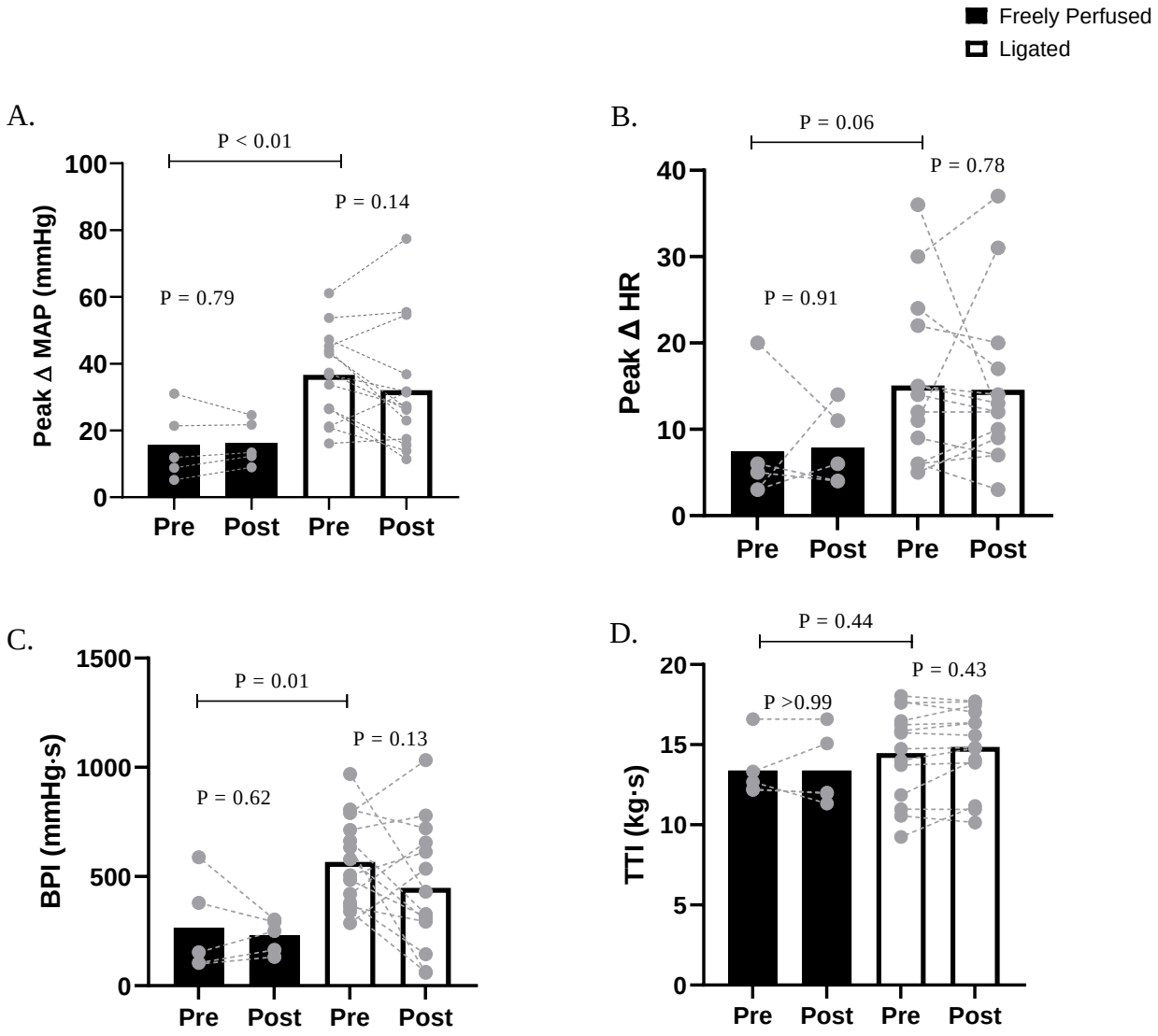


**Figure 2: Cardiovascular response to hindlimb muscle stretch pre- and post-HOE-140 administration in freely perfused and ligated rats**

The peak ΔMAP (A) and BPI (C) were significantly elevated in the ligated group compared with the freely perfused group. Peak ΔHR (B) trended toward being significantly higher in ligated rats. HOE-140 had no effect on either the freely perfused or the ligated groups on the peak ΔMAP, BPI, and peak ΔHR response to stretch. TTI (D) was not significantly different between conditions or across groups. Data were compared with paired or unpaired Student's t-tests.

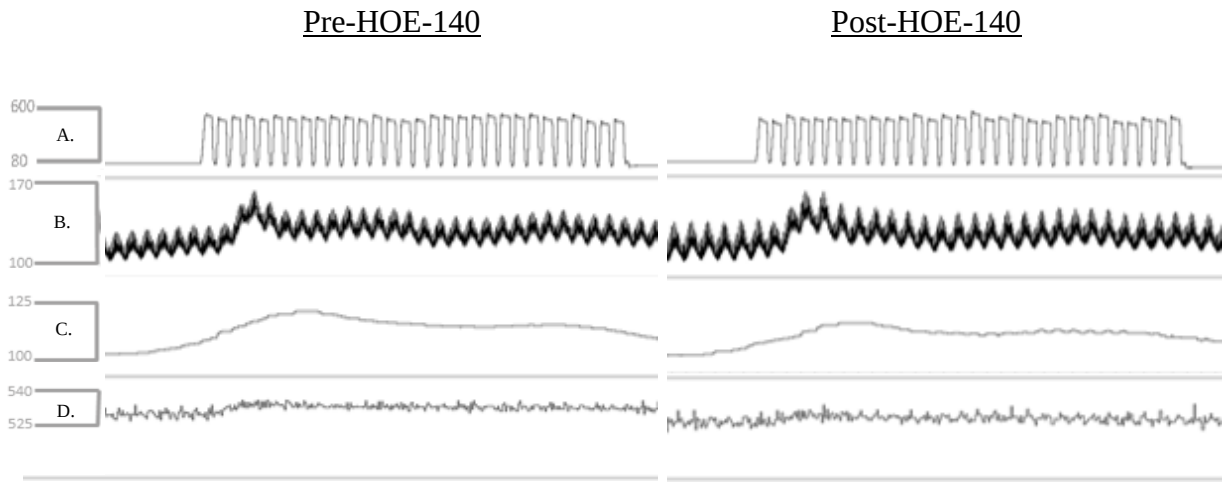


**Figure 3: Original images of hindlimb muscle stretch pre- and post-HOE-140 administration in a ligated rat**

Original tracing of the tension development (A; g) and BP (B; mmHg), MAP (C; mmHg), and HR (D; bpm) response to dynamic hindlimb muscle stretch in a ligated rat pre- and post-HOE-140 administration.

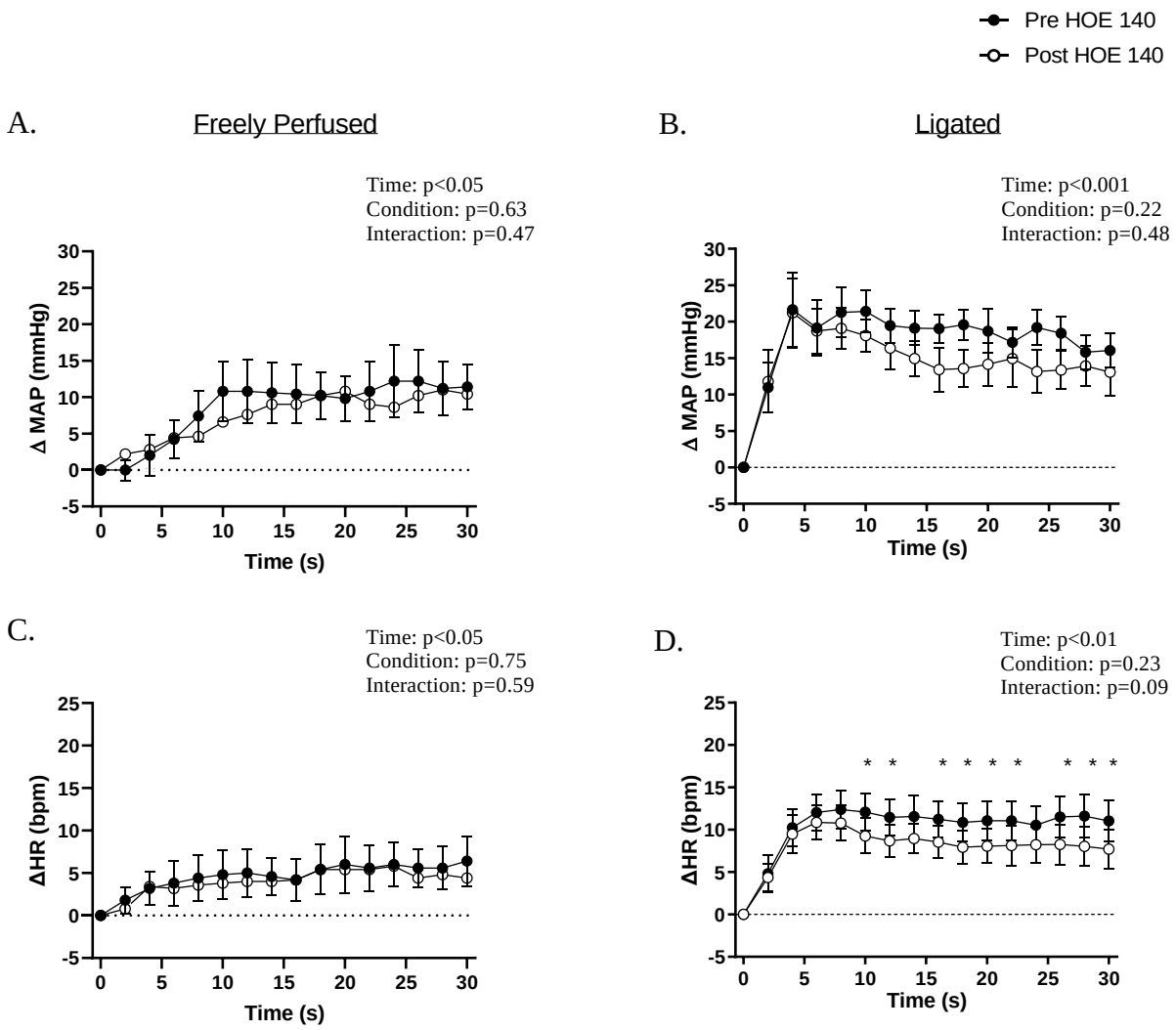


**Figure 4: Time course of the cardiovascular response to hindlimb muscle stretch in freely perfused and ligated rats pre- and post-HOE-140 administration**

In neither the freely perfused nor the ligated groups was there a significant main effect or interaction between conditions for either ΔMAP (panels A and B, respectively) or ΔHR (C and D) responses to stretch. However, at several time points the ligated group displayed a significantly reduced ΔHR response post-HOE-140. Data were compared with repeated measures two-way ANOVAs with Holm-Šidák multiple comparison tests. The dashed lines represent baseline values. *P < 0.05.

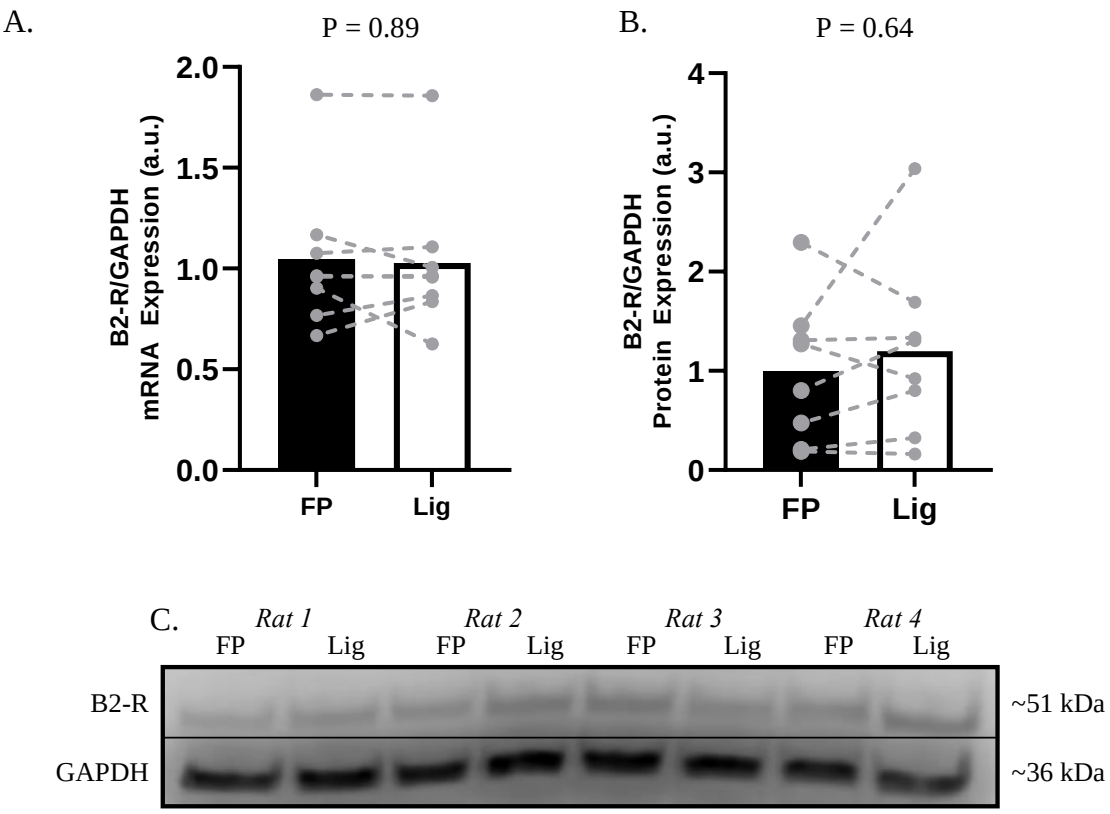


**Figure 5: B2-R expression in DRG of ligated and freely perfused hindlimbs**

Quantification of B2-R mRNA (A) and protein (B) expression was analyzed with paired Student's t-tests. There is no statistical difference between B2-R expression in the DRG associated with a ligated hindlimb and that of a freely perfused hindlimb. Visual representation of B2-R protein expression (C) shows no difference between the $L_4$ and $L_5$ DRG corresponding to ligated (Lig) hindlimbs and the $L_4$ and $L_5$ DRG corresponding to non-operated freely perfused (FP) hindlimbs. GAPDH was used as a protein loading control.

# Chapter 4 - Discussion

We investigated the role of B2-Rs in the chronic mechanoreflex sensitization in the rat model of simulated PAD in which a femoral artery is ligated for ~72 hours. After confirming that 100 ng of HOE-140 effectively blocked B2-R, we determined there is no significant effect of B2-R blockade on the pressor response to dynamic hindlimb skeletal muscle stretch in either the ligated or freely perfused group. Additionally, there was no effect of B2-R blockade on the cardioaccelerator response to stretch in the freely perfused group and only a modest effect at several time points during the stretch protocol in the ligated group. We further found that femoral artery ligation had no effect on $L_4$ and $L_5$ DRG B2-R protein or mRNA expression. We therefore conclude that B2-Rs do not play a crucial role in the chronic mechanoreflex sensitization in the rat model of simulated PAD. More specifically, B2-R activity is not required to produce the exaggerated pressor response to dynamic mechanoreflex activation in the rat model of simulated PAD. Our findings help to delineate the mechanisms of blood pressure control in the rat model of simulated PAD and therefore may have implications for blood pressure control in human PAD patients.

First confirming that the 100 ng of HOE-140 (~255 ng/kg) reduced the pressor response to BK injection into the arterial supply of the hindlimb was critical as this dose is markedly lower than that used to study the role of B2-R in the mechanoreflex and EPR in previous investigations (i.e, doses ranging from 2 to 20 μg/kg; Koba et al., 2010, Lu et al.. 2013, Pan, Stebbins & Longhurst, 1993). HOE-140 inhibits B2-Rs with an IC50 of 1.07 nM (Hock et al., 1991). With an estimated rat hindlimb circulation volume of 15 ml (Yamauchi et al., 2014) and assuming equal distribution within that volume, the HOE-140 concentration in the present experiments was approximately 5 nM. From these calculations, it is reasonable to assume

sufficient inhibition of B2-R by the 100 ng dose of HOE-140 used in the present investigation, an assumption confirmed here by the significant HOE-140-induced reduction in MAP response to BK injection. Furthermore, we have collected preliminary data showing that a dose of 500 ng (~1.3 μg/kg) injected into the jugular vein of ligated rats attenuates the pressor response to dynamic hindlimb skeletal muscle contraction (unpublished observations). Given that some HOE-140 injected into the arterial supply of the hindlimb is likely to circulate systemically, there exists the potential for a systemic or centrally active effect of the drug (Pan, Stebbins & Longhurst, 1993; Stebbins & Bonigut, 1996) when doses greater than or equal to 500 ng are used. For example, Lu et al. (2013) found that injection of 5 μg/kg of HOE-140, approximately 20 times the dose used presently, into the arterial supply of the hindlimb reduced the pressor response to static hindlimb muscle stretch in freely perfused and ligated rats but did not trap the injectate within the hindlimb circulation and did not perform systemic control experiments.

Consistent with our finding that the pressor response to dynamic stretch, a robust mechanical stimulus (Kempf et al., 2018), was not significantly affected by B2-R inhibition in the freely perfused condition, Koba et al. (2010) showed a similar finding of no effect of i.a. injection of HOE-140 (2 μg/kg) into the hindlimb circulation on the RSNA response to intermittent contraction in healthy rats. Leal et al. (2013) similarly showed no statistically significant effect of HOE-140 on the afferent response to dynamic contraction in healthy cats. However, Leal et al. (2013) found that group III afferent response to static contraction in healthy cats was reduced after HOE-140 administration. It is important to note here that group III thin fiber sensory afferents also respond to metabolic stimuli. The increased blood flow associated with dynamic contraction would produce a vascular bed “washout” of BK not seen in static contraction (Copp et al., 2015; Kruse, Selitte & Scheuermann, 2016), raising the possibility of a

role for B2-R activity in health in the metaboreflex rather than the mechanoreflex. However, static and dynamic stretch activate the mechanoreflex in isolation from contraction-induced metabolite production. Therefore, the role of B2-R on the sensory endings of thin fiber afferents in the mechanoreflex in healthy subjects (Stebbins & Longhurst 1986; Pan, Stebbins & Longhurst 1993) may be dependent on the mode of mechanoreflex activation.

Our finding that 100 ng of HOE-140 had no effect on the pressor response to dynamic hindlimb skeletal muscle stretch in the chronically (~72 hours) ligated condition is in contrast to the Lu et al. (2013) investigation which found that HOE-140 reduced the pressor response to static hindlimb skeletal muscle stretch in rats subjected to 24 hours of femoral artery ligation. A few explanations for this discrepancy exist. First, as previously detailed, Lu et al. (2013) used a comparatively large dose of HOE-140 without a blood flow occlusion and did not perform systemic control experiments. Therefore, the possibility of a systemic or centrally active effect of the drug in the Lu et al. investigation cannot be ruled out. Second, as described above, it is likely there are differences in B2-R regulation of the mechanoreflex during static and dynamic mechanoreflex activation modalities in health which may persist in the ligated condition. Third, Lu et al. (2013) allowed only 24 hours of exposure to femoral artery ligation to occur, and in this time frame they were able to report an increase in B2 receptor expression in the DRG innervating the injured limb. The rats used in the present investigation were exposed to femoral artery ligation for ~72 hours before DRG harvest took place. After this extended timeframe, we showed there was no difference in B2-R protein or mRNA expression. It is then possible B2-R expression, and subsequently B2-R regulation of the mechanoreflex, is dependent on the time of exposure to the femoral artery ligation injury. In contrast to the absence of significant effect of HOE-140 on the pressor response to dynamic stretch in the ligated group found presently, we

found modest but statistically significant effects of HOE-140 on the cardioaccelerator response. However, given the relative minute value of this reduction (see **Figure 4**) when compared with the magnitude of the baseline HR of >500 bpm in the rats in this investigation, the physiological importance of this may be negligible.

A surprising finding in the present investigation was the variability in the pressor response to stretch post-HOE-140 administration demonstrated between individual ligated rats (see individual values **Figure 2**). One possible explanation that may contribute to the lack of significant effect as well as the variability in the effect of HOE-140 in ligated rats is the complexity of downstream B2-R signaling in sensory neurons. While shown to mainly couple with $G_q$ subunits (Marceau et al., 2020), B2-R also couple to $G_i$ subunits (Leeb-Lundberg et al., 2005). $TxA_2$-R couple with $G_q$ subunits, and upon inhibition, a consistent reduction in pressor response in the ligated but not freely perfused rats is demonstrated (Rollins et al., 2020). When stimulated, these $G_q$ subunits activate inositol triphosphate signaling, resulting in increased cytosolic calcium levels and subsequently exaggerated mechanical sensitivity (Huang et al., 2004; Dubin et al., 2012). Endoperoxide 4 receptors (EP4-R) couple with $G_s$ subunits (Southall & Vasko, 2001), resulting in an increase in cyclic adenosine monophosphate (cAMP) and subsequent sensitization of sensory afferents (Southall & Vasko, 2001). B2-R coupling to $G_i$ subunits results in an opposing attenuation of cAMP (Leeb-Lundberg et al., 2005), and may be preventing potential roles for EP4-R in mechanoreflex sensitization. Because B2-R couple to both $G_q$ and $G_i$ subunits, it is likely their downstream actions are representative of this duality of cellular signaling capability.

A few experimental limitations merit mentioning. First, PAD develops slowly as a result of atherosclerotic plaque buildup in the vessels supplying the limbs (Muller et al., 1985),

whereas femoral artery ligation is a nearly instantaneous and comparatively short-term injury. However, the hemodynamic and blood pressure patterns of rats having undergone femoral artery ligation mimic those of human PAD patients with sufficient similarity as to allow valid comparison (Stone & Kaufman, 2015; Prior et al., 2004; Yang et al., 2000). Secondly, skeletal muscle stretch passively lenthens the muscle, whereas contraction involves a shortening of the muscle with concomitant increases in intramuscular pressure (Gallagher et al 2001). However, the vast majority of the afferents that respond to rat hindlimb muscle stretch also respond to muscle contraction (Stone et al 2015). Therefore, the dynamic hindlimb skeletal muscle stretch mode of mechanoreflex activation is able to contribute importantly to the delineation of mechanisms of mechanoreflex activation. Finally, our study included only male rats. To date, no investigations into the translatability of male-specific research on the pathophysiological mechanisms of exercise pressor reflex exaggeration in PAD or a model thereof to the female population have been conducted. Therefore the findings herein cannot be presumed to apply ubiquitously to both sexes.

In conclusion, we tested the hypothesis that B2-R blockade in male rats subjected to femoral artery ligation would result in a greater attenuation of the pressor response to dynamic hindlimb muscle stretch than in rats subjected to a sham procedure. The results of the present study did not support our hypothesis and instead indicate that the BK-B2-R interaction is not necessary for the chronic mechanoreflex sensitization present in the rat model of simulated PAD. It is important to note that our results do not completely rule out possible involvement of B2-R in the exaggerated mechanoreflex in ligated rats given the complex nature and inherent redundancy of second messenger signaling associated with G protein coupled receptors. The findings herein help to progress the understanding of the mechanisms responsible for chronic mechanoreflex

sensitization in the rat model of simulated PAD in which a femoral artery is chronically ligated which may extend to human PAD patients.

# Chapter 5 - References

Åneman A, Pontén J, Fändriks L, Eisenhofer G, Friberg P, Biber B. Splanchnic and renal sympathetic activity in relation to hemodynamics during isoflurane administration in pigs. Anesth Analg. 1995 Jan;80(1):135-42. doi: 10.1097/00000539-199501000-00023. PMID: 7802270.

Baccelli G, Reggiani P, Mattioli A, Corbellini E, Garducci S, Catalano M. The exercise pressor reflex and changes in radial arterial pressure and heart rate during walking in patients with arteriosclerosis obliterans. Angiology. 1999 May;50(5):361-74. doi: 10.1177/000331979905000502. PMID: 10348424.

Bakke EF, Hisdal J, Jørgensen JJ, Kroese A, Stranden E. Blood pressure in patients with intermittent claudication increases continuously during walking. Eur J Vasc Endovasc Surg. 2007 Jan;33(1):20-5. doi: 10.1016/j.ejvs.2006.06.023. Epub 2006 Aug 23. PMID: 16934498.

Butenas ALE, Hopkins TD, Rollins KS, Felice KP, Copp SW. Investigation of the mechanisms of cyclooxygenase-mediated mechanoreflex sensitization in a rat model of simulated peripheral artery disease. Am J Physiol Heart Circ Physiol. 2019 Nov 1;317(5):H1050-H1061. doi: 10.1152/ajpheart.00399.2019. Epub 2019 Aug 30. PMID: 31469294; PMCID: PMC6879919.

Copp SW, Kim JS, Ruiz-Velasco V, Kaufman MP. The mechano-gated channel inhibitor GsMTx4 reduces the exercise pressor reflex in decerebrate rats. J Physiol. 2016 Feb 1;594(3):641-55. doi: 10.1113/JP271714. Epub 2016 Jan 5. PMID: 26608396; PMCID: PMC4930067.

Copp SW, Stone AJ, Li J, Kaufman MP. Role played by interleukin-6 in evoking the exercise pressor reflex in decerebrate rats: effect of femoral artery ligation. Am J Physiol Heart Circ Physiol. 2015 Jul 1;309(1):H166-73. doi: 10.1152/ajpheart.00195.2015. Epub 2015 Apr 24. PMID: 25910806; PMCID: PMC4491520.

de Liefde II, Hoeks SE, van Gestel YR, Bax JJ, Klein J, van Domburg RT, Poldermans D. Usefulness of hypertensive blood pressure response during a single-stage exercise test to predict long-term outcome in patients with peripheral arterial disease. Am J Cardiol. 2008 Oct 1;102(7):921-6. doi: 10.1016/j.amjcard.2008.05.032. Epub 2008 Jul 9. PMID: 18805123.

Dubin AE, Schmidt M, Mathur J, Petrus MJ, Xiao B, Coste B, Patapoutian A. Inflammatory signals enhance piezo2-mediated mechanosensitive currents. Cell Rep. 2012 Sep 27;2(3):511-7. doi: 10.1016/j.celrep.2012.07.014. Epub 2012 Aug 23. PMID: 22921401; PMCID: PMC3462303.

Gallagher KM, Fadel PJ, Smith SA, Norton KH, Querry RG, Olivencia-Yurvati A, Raven PB. Increases in intramuscular pressure raise arterial blood pressure during dynamic exercise. J Appl Physiol (1985). 2001 Nov;91(5):2351-8. doi: 10.1152/jappl.2001.91.5.2351. PMID: 11641380.

Hayes SG, McCord JL, Koba S, Kaufman MP. Gadolinium inhibits group III but not group IV muscle afferent responses to dynamic exercise. J Physiol. 2009 Feb 15;587(Pt 4):873-82. doi: 10.1113/jphysiol.2008.164640. Epub 2008 Dec 22. PMID: 19103679; PMCID: PMC2669976.

Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke S, Breipohl G, König W, Knolle J, et al. Hoe 140 a new potent and long acting bradykinin-antagonist: in vitro

studies. Br J Pharmacol. 1991 Mar;102(3):769-73. doi: 10.1111/j.1476-5381.1991.tb12248.x. PMID: 1364851; PMCID: PMC1917958.

Huang JS, Ramamurthy SK, Lin X, Le Breton GC. Cell signalling through thromboxane A2 receptors. Cell Signal. 2004 May;16(5):521-33. doi: 10.1016/j.cellsig.2003.10.008. PMID: 14751539.

Kempf EA, Rollins KS, Hopkins TD, Butenas AL, Santin JM, Smith JR, Copp SW. Chronic femoral artery ligation exaggerates the pressor and sympathetic nerve responses during dynamic skeletal muscle stretch in decerebrate rats. Am J Physiol Heart Circ Physiol. 2018 Feb 1;314(2):H246-H254. doi: 10.1152/ajpheart.00498.2017. Epub 2017 Oct 20. PMID: 29054973.

Koba S, Xing J, Sinoway LI, Li J. Bradykinin receptor blockade reduces sympathetic nerve response to muscle contraction in rats with ischemic heart failure. Am J Physiol Heart Circ Physiol. 2010 May;298(5):H1438-44. doi: 10.1152/ajpheart.00558.2009. Epub 2010 Mar 5. PMID: 20207818; PMCID: PMC2867438.

Kruse NT, Silette CR, Scheuermann BW. Influence of passive stretch on muscle blood flow, oxygenation and central cardiovascular responses in healthy young males. Am J Physiol Heart Circ Physiol. 2016 May 1;310(9):H1210-21. doi: 10.1152/ajpheart.00732.2015. Epub 2016 Mar 4. PMID: 26945077.

Leal AK, Stone AJ, Yamauchi K, McCord JL, Kaufman MP. Blockade of B2 receptors attenuates the responses of group III afferents to static contraction. Neurosci Lett. 2013 Oct 25;555:231-6. doi: 10.1016/j.neulet.2013.09.013. Epub 2013 Sep 12. PMID: 24036460; PMCID: PMC3842220.

Leeb-Lundberg LM, Marceau F, Müller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. Pharmacol Rev. 2005 Mar;57(1):27-77. doi: 10.1124/pr.57.1.2. PMID: 15734727.

Lu J, Xing J, Li J. Bradykinin B2 receptor contributes to the exaggerated muscle mechanoreflex in rats with femoral artery occlusion. Am J Physiol Heart Circ Physiol. 2013 Apr 15;304(8):H1166-74. doi: 10.1152/ajpheart.00926.2012. Epub 2013 Feb 15. PMID: 23417862; PMCID: PMC3625911.

Marceau F, Bachelard H, Bouthillier J, Fortin JP, Morissette G, Bawolak MT, Charest-Morin X, Gera L. Bradykinin receptors: Agonists, antagonists, expression, signaling, and adaptation to sustained stimulation. Int Immunopharmacol. 2020 Feb 24;82:106305. doi: 10.1016/j.intimp.2020.106305. Epub ahead of print. PMID: 32106060.

McCord JL, Kaufman MP. Reflex Autonomic Responses Evoked by Group III and IV Muscle Afferents. In: Kruger L, Light AR, editors. Translational Pain Research: From Mouse to Man. Boca Raton (FL): CRC Press/Taylor & Francis; 2010. Chapter 12. PMID: 21882465.

McCloskey DI, Mitchell JH. The use of differential nerve blocking techniques to show that the cardiovascular and respiratory reflexes originating in exercising muscle are not mediated by large myelinated afferents. J Physiol. 1972 Apr;222(1):50P-51P. PMID: 5037091.

Mense S. Nervous outflow from skeletal muscle following chemical noxious stimulation. J Physiol. 1977 May;267(1):75-88. doi: 10.1113/jphysiol.1977.sp011802. PMID: 141496; PMCID: PMC1283603.

Mense S, Stahnke M. Responses in muscle afferent fibres of slow conduction velocity to contractions and ischaemia in the cat. J Physiol. 1983 Sep;342:383-97. doi: 10.1113/jphysiol.1983.sp014857. PMID: 6631740; PMCID: PMC1193965.

Mitchell JH, Kaufman MP, Iwamoto GA. The exercise pressor reflex: its cardiovascular effects, afferent mechanisms, and central pathways. Annu Rev Physiol. 1983;45:229-42. doi: 10.1146/annurev.ph.45.030183.001305. PMID: 6342515.

Muller MD, Drew RC, Blaha CA, Mast JL, Cui J, Reed AB, Sinoway LI. Oxidative stress contributes to the augmented exercise pressor reflex in peripheral arterial disease patients. J Physiol. 2012 Dec 1;590(23):6237-46. doi: 10.1113/jphysiol.2012.241281. Epub 2012 Sep 24. PMID: 23006479; PMCID: PMC3530129.

Muller MD, Reed AB, Leuenberger UA, Sinoway LI. Physiology in medicine: peripheral arterial disease. J Appl Physiol (1985). 2013 Nov 1;115(9):1219-26. doi: 10.1152/japplphysiol.00885.2013. Epub 2013 Aug 22. PMID: 23970534; PMCID: PMC3841832.

Muller MD, Drew RC, Ross AJ, Blaha CA, Cauffman AE, Kaufman MP, Sinoway LI. Inhibition of cyclooxygenase attenuates the blood pressure response to plantar flexion exercise in peripheral arterial disease. Am J Physiol Heart Circ Physiol. 2015 Aug 1;309(3):H523-8. doi: 10.1152/ajpheart.00267.2015. Epub 2015 Jun 8. PMID: 26055794; PMCID: PMC4525087.

Pan HL, Stebbins CL, Longhurst JC. Bradykinin contributes to the exercise pressor reflex: mechanism of action. J Appl Physiol (1985). 1993 Nov;75(5):2061-8. doi: 10.1152/jappl.1993.75.5.2061. PMID: 8307860.

Prior BM, Lloyd PG, Ren J, Li H, Yang HT, Laughlin MH, Terjung RL. Time course of changes in collateral blood flow and isolated vessel size and gene expression after femoral artery occlusion in rats. Am J Physiol Heart Circ Physiol. 2004 Dec;287(6):H2434-47. doi: 10.1152/ajpheart.00398.2004. Epub 2004 Jul 22. PMID: 15271665.

Rollins KS, Butenas ALE, Felice KP, Matney JE, Williams AC, Kleweno TE, Copp SW. Thromboxane A2 receptors mediate chronic mechanoreflex sensitization in a rat model of simulated peripheral artery disease. Am J Physiol Heart Circ Physiol. 2020 Aug 1;319(2):H320-H330. doi: 10.1152/ajpheart.00255.2020. Epub 2020 Jun 12. PMID: 32530751; PMCID: PMC7473920.

Rollins KS, Hopkins TD, Butenas AL, Felice KP, Ade CJ, Copp SW. Cyclooxygenase inhibition does not impact the pressor response during static or dynamic mechanoreflex activation in healthy decerebrate rats. Am J Physiol Regul Integr Comp Physiol. 2019 Sep 1;317(3):R369-R378. doi: 10.1152/ajpregu.00080.2019. Epub 2019 Jun 26. PMID: 31241976; PMCID: PMC6766704.

Rollins KS, Smith JR, Esau PJ, Kempf EA, Hopkins TD, Copp SW. Bradykinin does not acutely sensitize the reflex pressor response during hindlimb skeletal muscle stretch in decerebrate rats. Am J Physiol Regul Integr Comp Physiol. 2017 Oct 1;313(4):R463-R472. doi: 10.1152/ajpregu.00187.2017. Epub 2017 Jul 19. PMID: 28724548.

Sapru HN, Krieger AJ. Procedure for the decerebration of the rat. Brain Res Bull. 1978 Nov-Dec;3(6):675-9. doi: 10.1016/0361-9230(78)90016-3. PMID: 318191.

Shu J, Santulli G. Update on peripheral artery disease: Epidemiology and evidence-based facts. Atherosclerosis. 2018 Aug;275:379-381. doi: 10.1016/j.atherosclerosis.2018.05.033. Epub 2018 May 22. PMID: 29843915; PMCID: PMC6113064.

Smith SA, Mitchell JH, Garry MG. Electrically induced static exercise elicits a pressor response in the decerebrate rat. J Physiol. 2001 Dec 15;537(Pt 3):961-70. doi: 10.1111/j.1469-7793.2001.00961.x. PMID: 11744768; PMCID: PMC2278979.

Southall MD, Vasko MR. Prostaglandin receptor subtypes, EP3C and EP4, mediate the prostaglandin E2-induced cAMP production and sensitization of sensory neurons. J Biol Chem. 2001 May 11;276(19):16083-91. doi: 10.1074/jbc.M011408200. Epub 2001 Feb 14. PMID: 11278900.

Stebbins CL, Bonigut S. Endogenous bradykinin in the thoracic spinal cord contributes to the exercise pressor reflex. J Appl Physiol (1985). 1996 Sep;81(3):1288-94. doi: 10.1152/jappl.1996.81.3.1288. PMID: 8889765.

Stebbins CL, Carretero OA, Mindroiu T, Longhurst JC. Bradykinin release from contracting skeletal muscle of the cat. J Appl Physiol (1985). 1990 Oct;69(4):1225-30. doi: 10.1152/jappl.1990.69.4.1225. PMID: 2124582.

Stebbins CL, Longhurst JC. Bradykinin in reflex cardiovascular responses to static muscular contraction. J Appl Physiol (1985). 1986 Jul;61(1):271-9. doi: 10.1152/jappl.1986.61.1.271. PMID: 2426240.

Stone AJ, Kaufman MP. The exercise pressor reflex and peripheral artery disease. Auton Neurosci. 2015 Mar;188:69-73. doi: 10.1016/j.autneu.2014.10.014. Epub 2014 Oct 22. PMID: 25458431; PMCID: PMC4336605.

Stone AJ, Copp SW, McCord JL, Kaufman MP. Femoral artery ligation increases the responses of thin-fiber muscle afferents to contraction. J Neurophysiol. 2015 Jun 1;113(10):3961-6. doi: 10.1152/jn.00288.2015. Epub 2015 Apr 8. PMID: 25855694; PMCID: PMC4485769.

Tsuchimochi H, McCord JL, Hayes SG, Koba S, Kaufman MP. Chronic femoral artery occlusion augments exercise pressor reflex in decerebrated rats. Am J Physiol Heart Circ Physiol. 2010 Jul;299(1):H106-13. doi: 10.1152/ajpheart.00141.2010. Epub 2010 Apr 23. PMID: 20418475; PMCID: PMC2904125.

Yamauchi K, Stone AJ, Kaufman MP. Hindlimb venous distention evokes a pressor reflex in decerebrated rats. Physiol Rep. 2014 Jun 6;2(6):e12036. doi: 10.14814/phy2.12036. PMID: 24907299; PMCID: PMC4208660.

Yang HT, Feng Y, Allen LA, Protter A, Terjung RL. Efficacy and specificity of bFGF increased collateral flow in experimental peripheral arterial insufficiency. Am J Physiol Heart Circ Physiol. 2000 Jun;278(6):H1966-73. doi: 10.1152/ajpheart.2000.278.6.H1966. PMID: 10843895.