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

by

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Abstract

Hindlimb skeletal muscle stretch (i.e., selective activation of the muscle mechanoreflex) in decerebrate rats evokes reflex increases in blood pressure and sympathetic nerve activity. Bradykinin has been found to sensitize mechano-gated channels through a bradykinin B2 receptor-dependent mechanism. Moreover, bradykinin B2 receptor expression on sensory neurons is increased following chronic femoral artery ligation in the rat (a model of simulated peripheral artery disease). We tested the hypothesis that, in decerebrate, unanesthetized rats, the injection of bradykinin into the arterial supply of a hindlimb would acutely augment (i.e., sensitize) the increase in blood pressure and renal sympathetic nerve activity (RSNA) during hindlimb muscle stretch to a greater extent in rats with a ligated femoral artery than in rats with freely perfused femoral arteries. The pressor response during static hindlimb muscle stretch was compared before and after the hindlimb arterial injection of 0.5 μg of bradykinin. The injection of bradykinin itself increased blood pressure to a greater extent in "ligated" rats (n=10) than in "freely perfused" rats (n=10). The increase in blood pressure during hindlimb muscle stretch, however, was not different before compared to after bradykinin injection in either freely perfused (control: 14±2, post-bradykinin: 15±2 mmHg, p=0.62) or ligated (control: 15±3, post-bradykinin: 14±2 mmHg, p=0.80) rats. Likewise, the increase in RSNA during stretch was not different before compared to after bradykinin injection in either group of rats. We conclude that bradykinin did not acutely sensitize the pressor response during hindlimb skeletal muscle stretch in either freely perfused or ligated decerebrate rats.

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Chapter 1 - Introduction

Mechanical and metabolic stimuli arising from within contracting skeletal muscles reflexly increase sympathetic nerve activity, blood pressure, and heart rate (8). The afferent arm of this reflex, termed the exercise pressor reflex (22), is comprised of group III and IV thin fiber afferents whose sensory endings are located within skeletal muscles and respond primarily to the mechanical and metabolic stimuli, respectively. The exercise pressor reflex is exaggerated in patients with various forms of cardiovascular disease and cardiovascular disease models compared to healthy counterparts (28, 40). For example, the exercise pressor reflex was greater in peripheral artery disease (PAD) patients compared to that found in aged-matched healthy control subjects (1-3, 25, 26). The exercise pressor reflex was also greater when evoked from a rat hindlimb in which the femoral artery was ligated ~72 hours before the experiment (a model of simulated PAD) compared to that evoked from the contralateral freely perfused hindlimb (41).

Bradykinin is an autocoid that is synthesized during skeletal muscle contraction and this contraction-induced synthesis is augmented by limb ischemia (36). Bradykinin has been found to play a role in activating the exercise pressor reflex. For example, the injection of bradykinin into the arterial supply of the hindlimb of animals has been shown to increase group III and IV thin fiber afferent responsiveness (9, 10, 17) and reflexly increase blood pressure (37). Moreover, both the inhibition of bradykinin synthesis (38) as well as bradykinin B2 receptor blockade (29) reduced the magnitude of the exercise pressor reflex. In addition to directly activating thin fiber muscle afferents, bradykinin has been shown to sensitize muscle afferent responsiveness to mechanical and metabolic stimuli (18, 19, 45). In rats with heart failure, for example, Xing et al. (45) found that the hindlimb arterial injection of bradykinin acutely augmented the pressor response evoked by the hindlimb arterial injection of α,β-methylene ATP.

Lu et al. (14) found that bradykinin B2 receptor blockade reduced the increase in blood pressure during static rat hindlimb skeletal muscle stretch (i.e., the muscle mechanoreflex); a maneuver that is commonly used to gain insight into the mechanical stimuli associated with muscle contraction apart from any metabolic stimuli (35). Moreover, the increase in blood pressure during muscle stretch was reduced following bradykinin B2 receptor blockade to a greater extent in rats in which a femoral artery was previously ligated compared to rats with freely perfused femoral arteries (14). The greater reduction in the pressor response was associated with a ligation-induced increase in lumbar dorsal root ganglia (DRG) bradykinin B2 receptor expression. That finding suggests that femoral artery ligation chronically sensitized the muscle mechanoreflex through a bradykinin B2 receptor-dependent mechanism. Additionally, Dubin et al. (6) found that bradykinin acutely sensitized piezo 2-mediated mechanically-activated currents in transfected HEK cells through a bradykinin B2 receptor-dependent mechanism and piezo channels have been suggested to contribute to the generation of the muscle mechanoreflex in rats with ligated femoral arteries (4). Whether bradykinin is capable of acutely sensitizing the muscle mechanoreflex thus augmenting the increase in blood pressure during hindlimb skeletal muscle stretch in rats with ligated femoral arteries is unknown.

Based on the findings summarized above, in decerebrate, unanesthetized rats we tested the hypotheses that: 1) bradykinin would increase blood pressure to a greater extent when injected into a hindlimb in which the femoral artery was ligated ~72 hours before the experiment compared to one in which the femoral artery was freely perfused, and 2) the hindlimb arterial injection of bradykinin would acutely augment (i.e., sensitize) the increase in blood pressure and renal sympathetic nerve activity (RSNA) during static triceps surae muscle stretch to a greater

extent in a hindlimb in which the femoral artery was previously ligated compared to one in which the femoral artery was freely perfused.

Chapter 2 - Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on young adult (average age: ~12 weeks, age range: 10-14 weeks) male Sprague-Dawley rats (n=44, average body weight: 399±8 g, range: 339-528 g; Charles River Laboratories). The rats were housed two per cage in accredited temperature and ventilation-controlled facilities with a 12/12 hour light/dark cycle (7 am/7 pm) and standard rat chow and water available *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.

Surgical procedures. 34 of the 44 rats used in this study had their left femoral artery ligated ~72 hours before the experiment. Specifically, these 34 rats were anesthetized with 3-4% isoflurane (balance O_2) and their left femoral artery was surgically exposed and ligated (5-0 suture) ~3 mm distal to the inguinal ligament. The incision was closed, and meloxicam (1 mg/kg sc) was administered as an analgesic. The rats recovered for ≥72 h before the initiation of the experimental protocol.

On the day of the experiment, all 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) 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 and measurement of arterial blood pressure (Physiological Pressure Transducer, AD Instruments), respectively. In all rats, the left calcaneus bone was

severed and the triceps surae (gastrocnemius, soleus and plantaris) muscles were exposed and linked by string to a force transducer (Grass FT03), which was attached to a rack-and-pinion. In 25 rats, bundles from the left renal sympathetic nerve were exposed using a retroperitoneal approach. The bundles were then glued (Kwik-Sil; World Precision Instruments) onto a pair of thin stainless steel recording electrodes, which were connected to a high impedance probe (Grass model HZP) and amplifier (Grass P511). Multi-unit signals from the renal sympathetic nerve fibers were filtered at 100 Hz (low frequency) and 1 kHz (high frequency). At the end of each experiment in which RSNA was measured, the ganglionic blocker hexamethonium bromide (20 mg/kg i.v.) was injected into the jugular vein to abolish nerve activity and the background noise was quantified.

In all rats used in protocol 1 (see below, 10 rats in which no femoral artery ligation was performed and 10 rats in which the left femoral artery was previously ligated), 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 (i.e. distal to the site of ligation in the hindlimbs in which the femoral artery was previously ligated). 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 all rats used in protocol 2 (see below, the left femoral artery was previously ligated in each of these 20 rats), the right calcaneus bone was also severed and the right triceps surae muscles exposed. In five of the 20 rats used in protocol 2, a PE-8 catheter was placed in both the right and left superficial epigastric arteries. In another 5 of the 20 rats used in protocol 2, both the right and left sciatic nerves were exposed so that shielded stimulating electrodes could be placed under the nerves to evoke hindlimb muscle contraction.

After the initial surgical procedures, all rats were placed in a Kopf stereotaxic frame and spinal unit with clamps placed around the pelvis and rostral lumbar vertebrae. 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 rats were given at least 60 minutes to recover prior to the initiation of any experimental protocol. The decerebration procedure was performed because anesthesia has been shown to depress the exercise pressor reflex in this species (34). Core temperature was measured by a rectal probe and maintained at ~37-38°C by an automated heating system (Harvard Apparatus). End-tidal CO_2 was monitored (Kent Scientific) continuously and maintained at ~38-40 mmHg by adjusting ventilation.

Experimental protocols. *Protocol 1 – Effect of bradykinin on the muscle mechanoreflex:* We compared the pressor response that resulted from the injection of bradykinin into the arterial supply of the hindlimb of "freely perfused" rats (n=10) and rats in which the femoral artery was previously ligated ("ligated" rats, n=10). Within each group of rats, we also compared the pressor response during static triceps surae muscle stretch before and after bradykinin injection. RSNA was measured in seven freely perfused rats and eight ligated rats. To evoke the muscle mechanoreflex, triceps surae muscle tension was set at ~100 g and baseline data were collected for 30 seconds. The left triceps surae muscles were then stretched for 30 seconds by rapidly turning the rack and pinion (see examples of the increase in blood pressure and RSNA during stretch in Figure 1). Following a ~5 minute recovery period from this control stretch maneuver, the snare on the left iliac artery and vein was tightened and 0.5 µg of bradykinin (dissolved in 0.2 ml of saline) was injected as a bolus (over ~2 s) into the arterial supply of the hindlimb via the

superficial epigastric artery catheter. Two minutes after the bradykinin injection, the snare was released and the hindlimb was allowed to reperfuse for an additional two minutes. The stretch protocol was then repeated. Evans blue dye was then injected into the superficial epigastric artery in the same manner as bradykinin to confirm that the injectate had access to the triceps surae muscle circulation. The triceps surae muscles stained blue in all experiments.

In four rats in which the left femoral artery was previously ligated, 20 μg/kg of α,β-methylene ATP was injected into the arterial supply of the hindlimb before and after the injection of 0.5 μg/kg bradykinin into the arterial supply of the hindlimb. The dose and method of bradykinin injection in these experiments was identical to that described above. This was done to confirm that the effects of bradykinin, at least in regards to its ability to sensitize purinergic receptors, were still present four minutes after its injection.

The dose of bradykinin was selected based on the fact that the injection of 0.5-1.5 μg/kg of bradykinin augmented the pressor response that resulted from the hindlimb arterial injection of α,β-methylene ATP in decerebrate rats (45), and that a range of 100 ng to 1 μg of bradykinin increased blood pressure when injected into the hindlimb arterial supply of the cat (29, 37). If one assumes a rat hindlimb volume of ~20 ml (47) and that the injection of 0.5 μg of bradykinin into the hindlimb arterial supply resulted in its distribution evenly throughout this volume, the resultant concentration of bradykinin within the hindlimb would be ~24 nM. The physiological concentrations of bradykinin in rat triceps surae muscle venous effluent blood at rest and during contractions are ~28 and 37 pM, respectively (calculated based on data from Stebbins et al. (36)). Importantly, we did not find a sensitizing effect of 0.5 μg of bradykinin on the muscle mechanoreflex (see results) so we did not feel it was necessary to reduce the dose of bradykinin closer to the physiological range. Moreover, we did not feel that it was necessary to increase the

dose of bradykinin to even greater supraphysiological levels. Regarding the timing of the post-bradykinin injection stretch maneuver (i.e., four minutes post-bradykinin injection), we performed it in as close temporal proximity as possible to the injection while avoiding the confounding effects of increased blood pressure resulting from the bradykinin injection itself. Put more simply, we waited until blood pressure returned to pre-injection levels and stabilized before initiating the post-bradykinin stretch.

Protocol 2 - Effect of ligation on the muscle mechanoreflex: In protocol 1, we found no difference in the pressor response during stretch between a ligated and freely perfused hindlimb when this comparison was made between different groups of rats. To investigate further, in 10 rats, we made within-rat comparisons of the pressor response during static triceps surae muscle stretch between a previously ligated hindlimb and the contralateral freely perfused hindlimb. RSNA was measured in seven of these 10 rats. Static triceps surae muscle stretch of the ligated and contralateral freely perfused hindlimbs were separated by ~5 minutes and were performed in random order.

In another group of five rats, we made within-rat comparisons of the pressor response to static triceps surae muscle contraction and stretch between a ligated and the contralateral freely perfused hindlimb. The stretch maneuvers were performed first, in random order, followed by the static contraction maneuvers, which were also performed in random order. The stretch maneuvers were performed exactly as described above. For static contractions, baseline muscle tension was set at ~100 g and baseline data were collected for 30 s. The sciatic nerve was then stimulated (40 Hz, 0.01 ms pulse duration, ≤2x motor threshold) with shielded electrodes for 30 s, which produced static hindlimb muscle contraction. After the contractions, rats were paralyzed with pancuronium bromide (1 mg/kg i.v.) and the sciatic nerves were stimulated with

the same parameters as those used to induce muscle contraction. This was done to ensure that the pressor responses to contraction were not the result of electrical activation of the axons of thin fiber afferents in the sciatic nerves. Stimulation of the sciatic nerves after pancuronium bromide injection did not increase blood pressure in any of the experiments.

In an additional group of five rats, we made within-rat comparisons of the pressor response to the injection of lactic acid into the arterial supply of the hindlimb and to triceps surae muscle stretch between the ligated and the contralateral freely perfused hindlimb. Before initiating the protocol, the rats were paralyzed with pancuronium bromide (1 mg/kg i.v.). The stretch maneuvers were performed first, in random order, followed by the lactic acid injections, which were also performed in random order. The stretch maneuvers were performed exactly as described above. For lactic acid injection, 0.2 ml of 24 mM lactic acid was injected into the arterial supply of the hindlimbs via the superficial epigastric artery catheters. In two rats, we subsequently cut the right and left sciatic nerves and repeated the lactic acid injections. In these experiments, injecting lactic acid following the cutting of the sciatic nerves did not produce increases in blood pressure. This indicates that the pressor response to lactic acid injection when the sciatic nerves were intact was entirely reflex in origin. Evans blue dye was injected into the right and left superficial epigastric artery catheters in the same manner in which lactic acid was injected. In all five rats, we observed qualitatively similar staining of the triceps surae muscles of the freely perfused and ligated hindlimbs, which indicates that when lactic acid was injected it had equal access to the triceps surae muscle circulations of the freely perfused and ligated hindlimbs.

Data analysis. Data were collected with a PowerLab and LabChart data acquisition system (AD Instruments). Arterial blood pressure, muscle tension, and RSNA were measured,

and mean arterial pressure (MAP) and heart rate (HR) were calculated and displayed in real time and recorded for offline analysis. The original RSNA data were rectified and corrected for the background noise measured following hexamethonium bromide injection (see Figure 1 for an example). RSNA signal-to-noise ratios were calculated for the baseline periods prior to muscle stretch using the noise measured following hexamethonium bromide injection. In protocol 1, baseline RSNA signal-to-noise ratios were not different between control and post-bradykinin conditions in either freely perfused (control: 1.9±0.6, post-bradykinin: 2.3±0.8, $p=0.31$) or ligated (control: 1.9±0.4 post-bradykinin: 1.9±0.3 $p=0.79$) rats. In protocol 2, baseline RSNA signal-to-noise ratios were not different between the freely perfused (2.3±0.3) and ligated hindlimb (2.3±0.5, $p=0.99$). Baseline MAP, HR, and RSNA were determined from the 30 s baseline periods that preceded each maneuver. The peak increase in MAP (peak Δ MAP) and HR during stretch, contraction, and/or bradykinin/lactic acid injection were calculated as the difference between the peak values wherever they occurred and their corresponding baseline value. Second-by-second time courses of the increase in MAP and RSNA were plotted as their change from baseline. The integral of the relative increase in RSNA during stretch (∫Δ RSNA, arbitrary units) was calculated by summing the Δ RSNA for the 30 s of stretch (23). The Δ tension-time index (Δ TTI) was calculated for stretch and contraction by integrating the area under the tension signal during the maneuver and subtracting the integrated area during the baseline period.

All values are expressed as mean±SEM. Data were compared with paired or unpaired Student's t-tests or mixed or two-way repeated measures ANOVAs with Holm-Sidak post hoc tests as appropriate. Statistical significance was accepted at $p<0.05$.

Chapter 3 - Results

Protocol 1: Effect of bradykinin on the muscle mechanoreflex

The injection of bradykinin into the arterial supply of a hindlimb produced marked increases in MAP. The increase in MAP was significantly greater (i.e., the increase was prolonged) when bradykinin was injected into the arterial supply of a ligated hindlimb compared to a freely perfused hindlimb (Figure 2, main effect for group $p=0.01$).

In freely perfused rats, there were no differences in the peak increase in MAP and HR, or $\int\Delta$ RSNA (Figure 3A and C, HR data are shown in Table 1) or the time course of the increase in MAP or RSNA (Figure 3B and D) between the control and post-bradykinin stretches. Likewise, in ligated rats, there were no differences in the peak increase in MAP and HR, or $\int\Delta$ RSNA (Figure 4A and C, Table 1) or the time course of the increase in MAP and RSNA between the control and post-bradykinin stretches (Figure 4B and D). By design, there were no differences in Δ TTI or the tension time course between the control and post-bradykinin stretches in either the freely perfused (Figure 3E and F) or ligated (Figure 4E and F) rats. In four additional ligated rats, the increase in MAP in response to the hindlimb arterial injection of α,β-methylene ATP was greater post-bradykinin compared to control (control: 23±10, post-bradykinin: 30±9 mmHg, $p=0.04$). The timing of the post-bradykinin α,β-methylene ATP injection was identical to the post-bradykinin stretch in the experiments described above.

In the control condition (i.e., before bradykinin injection), comparisons between the different groups of freely perfused and ligated rats revealed no difference in the peak increase in MAP (freely perfused: 14±2 and ligated: 15±3 mmHg, $p=0.81$) and a strong trend towards a difference in the $\int\Delta$ RSNA (freely perfused: 475±133 and ligated: 189±51 au, $p=0.055$) during stretch. Those findings prompted the experiments in protocol 2.

Protocol 2: Effect of ligation on the muscle mechanoreflex

In order to investigate the effect of femoral artery ligation on the muscle mechanoreflex more definitively than in protocol 1, we performed experiments in which we compared the increase in blood pressure and RSNA during stretch between a ligated hindlimb and the contralateral freely perfused hindlimb within the same rat. In 10 rats, there were no differences in the peak increase in MAP and HR, or ∫Δ RSNA (Figure 5A and C, Table 1) or the time course of the increase in MAP or RSNA (Figure 5B and D) during stretch between the ligated and the freely perfused hindlimb. By design, there were no differences in the Δ TTI or the tension time course of the stretches between the freely perfused and ligated hindlimb (Figure 5E and F). These findings in which the comparisons of the responses during stretch of freely perfused and ligated hindlimbs were made within the same rat confirmed our findings from protocol 1.

In five additional rats, the increase in MAP was significantly greater during static contraction of the ligated hindlimb compared to contraction of the contralateral freely perfused hindlimb (Figure 6A). In these same five rats, however, there was no difference in the pressor response during static stretch between hindlimbs (Figure 6C). There were no differences in the increase in HR during contraction or stretch between hindlimbs (Table 1). By design, there were no differences in the tension developed during the contraction (Figure 6B) and stretch (Figure 6D) between freely perfused and ligated hindlimbs.

In another group of five rats in which the left femoral artery was ligated and the right was freely perfused, the increase in MAP was significantly greater when lactic acid was injected into the arterial supply of the ligated hindlimb compared to the freely perfused hindlimb (Figure 7A). In these same five rats, however, there was no difference in the increase in MAP during stretch

between hindlimbs (Figure 7B). By design, there were no differences in the tension developed during stretch (Figure 7C) between freely perfused and ligated hindlimbs.

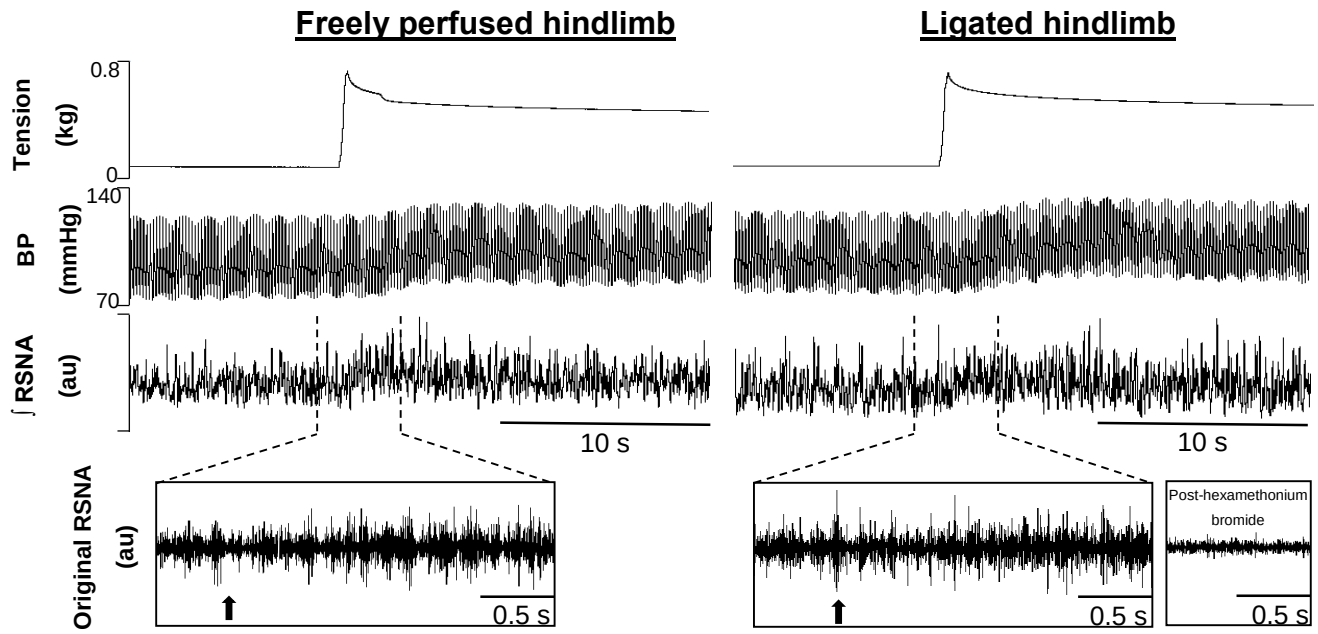

Figure 1: Original data of the pressor response to static muscle stretch

Original data from one rat showing the increase in blood pressure (BP) and integrated renal sympathetic nerve activity (∫ RSNA) before and during the first ~20 s of static triceps surae muscle stretch of the freely perfused hindlimb and the hindlimb in which the femoral artery was previously ligated. The insets show the original renal sympathetic nerve recording and background noise after injection of hexamethonium bromide. The arrows indicate the onset of stretch. The baseline RSNA signal-to-noise ratio calculated prior to stretch of the freely perfused hindlimb was 1.3 and prior to stretch of the ligated hindlimb was 1.2.

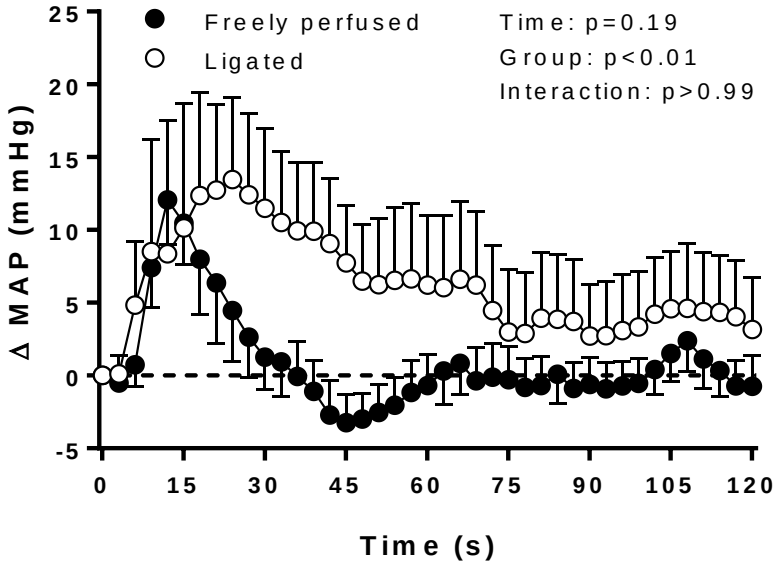

Figure 2: Pressor response to the arterial injection of bradykinin into the rat hindlimb

Bradykinin increased blood pressure to a greater extent (i.e., the increase was prolonged) when it was injected into the arterial supply of the hindlimb of rats in which the femoral artery was previously ligated (n=10, open circles) than it did when injected into the arterial supply of the hindlimb of rats in which the femoral artery was freely perfused (n=10, closed circles). Bradykinin (0.5 μg in 0.2 ml saline) injection was initiated at time "0" and lasted ~2 s. Data were analyzed with a mixed two-way ANOVA.

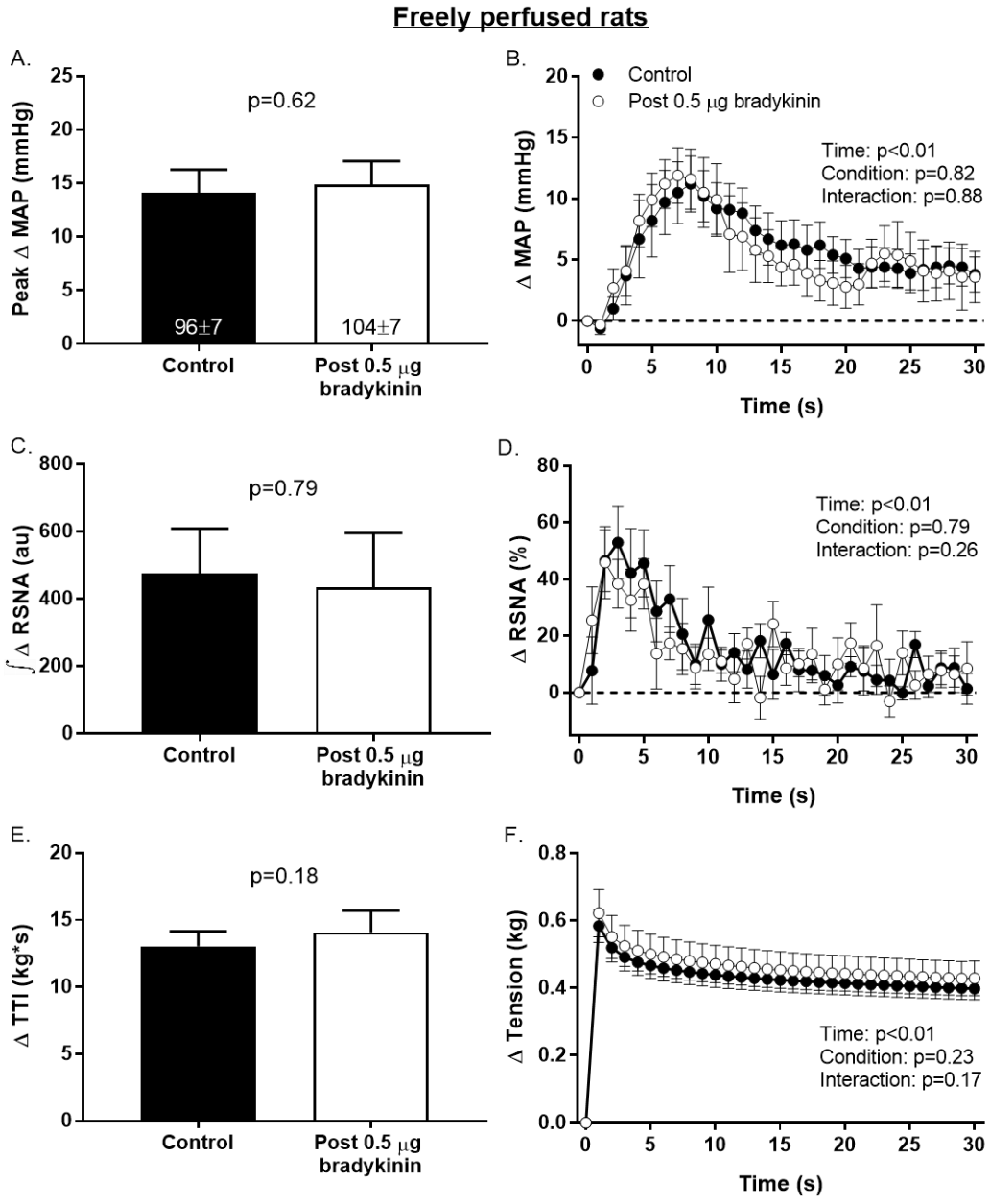

Figure 3: Static stretch in a freely perfused rat before and after bradykinin injection

In rats with freely perfused femoral arteries, there were no differences in the increase in mean arterial blood pressure (MAP, panels A and B) or renal sympathetic nerve activity (RSNA, panels C and D) during static triceps surae muscle stretch before (control) compared to after (post) bradykinin (0.5 μg) injection into the arterial supply of hindlimb (n=10 overall, n=7 for

RSNA). The tension time indexes (panel E) and tension time courses (panel F) were not different between the control and post-bradykinin stretches. Numbers within mean bars represent corresponding baseline values. Data were compared with paired Student's t-tests or repeated measures two-way ANOVAs as appropriate.

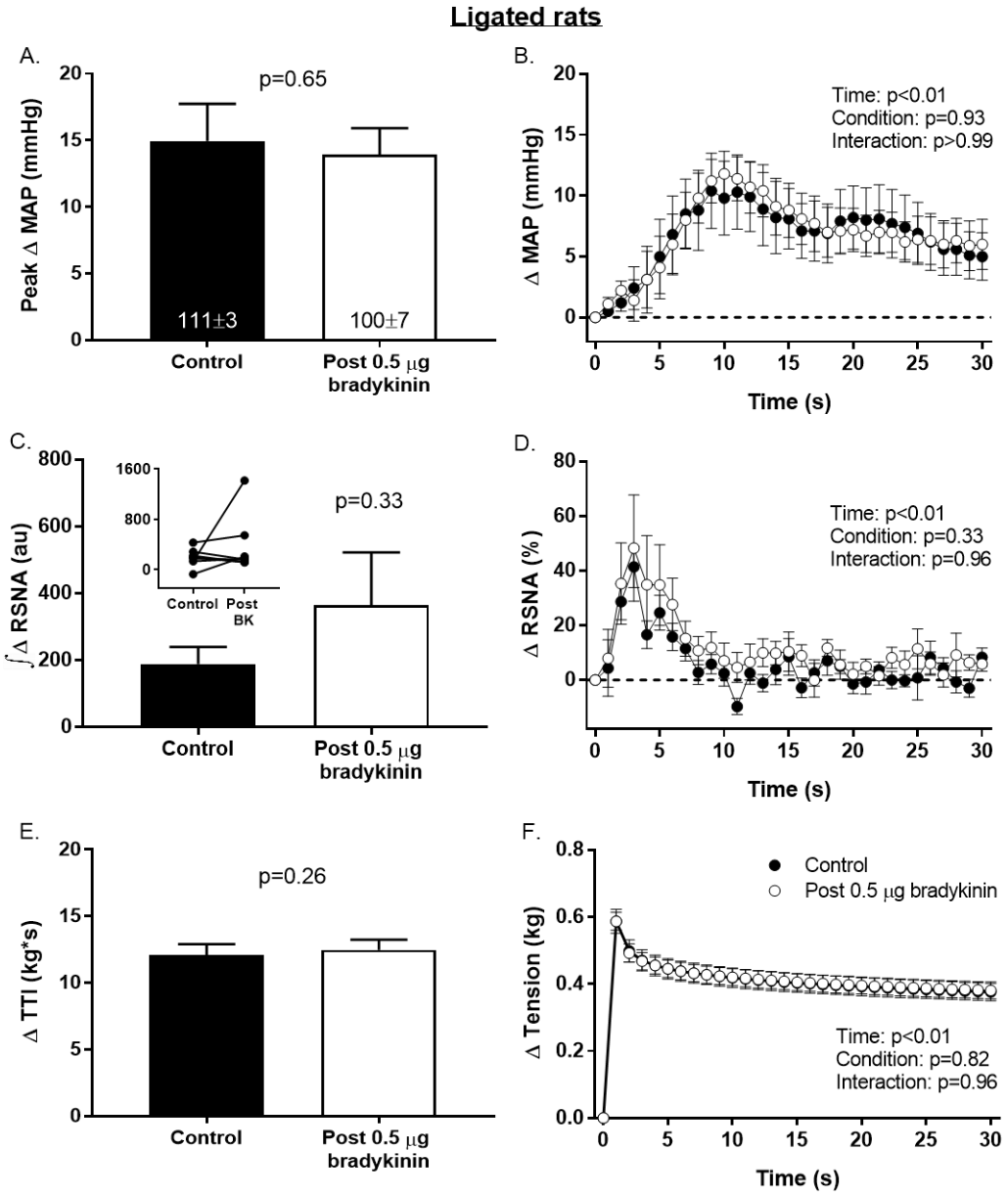

Figure 4: Static stretch in a ligated rat before and after bradykinin injection

In rats with a ligated femoral artery, there were no differences in the increase in mean arterial blood pressure (MAP, panels A and B) or renal sympathetic nerve activity (RSNA, panels C and D) during static triceps surae muscle stretch before (control) compared to after (post) bradykinin (0.5 μg) injection into the arterial supply of hindlimb (n=10 overall, n=8 for RSNA). The

tension time indexes (panel E) and tension time courses (panel F) were not different between the control and post-bradykinin stretches. The inset in Panel C shows individual $\int\Delta$ RSNA responses during stretch which demonstrates that the apparent (but not statistically significant) difference in the $\int\Delta$ RSNA between control and post-bradykinin conditions is attributable to one outlier response and was not a consistent finding. Numbers within mean bars represent corresponding baseline values. Data were compared with paired Student's t-tests or repeated measures two-way ANOVAs as appropriate.

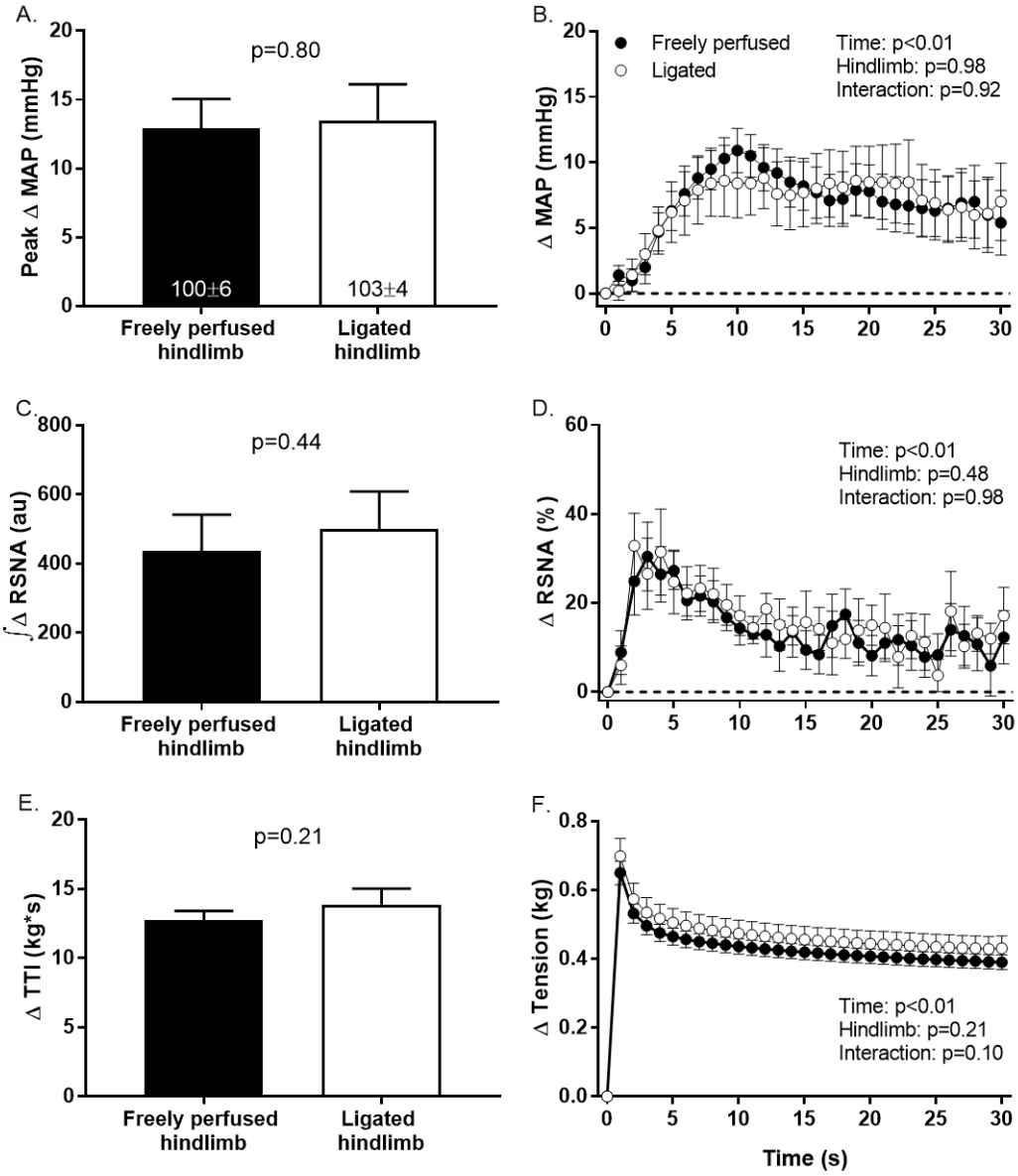

Figure 5: Pressor response during static stretch of freely perfused vs. ligated hindlimbs

In 10 rats, there were no differences in the increase in mean arterial blood pressure (MAP, panels A and B) or renal sympathetic nerve activity (RSNA, panels C and D) during static triceps surae muscle stretch of the hindlimb in which the femoral artery was ligated compared to the contralateral hindlimb in which the femoral artery was freely perfused (n=10 overall, n=7 for

RSNA). The tension time index (panel E) and tension time course (panel F) were not different between freely perfused and ligated hindlimbs. Numbers within mean bars represent corresponding baseline values. Data were compared with paired Student's t-tests or repeated measures two-way ANOVAs as appropriate.

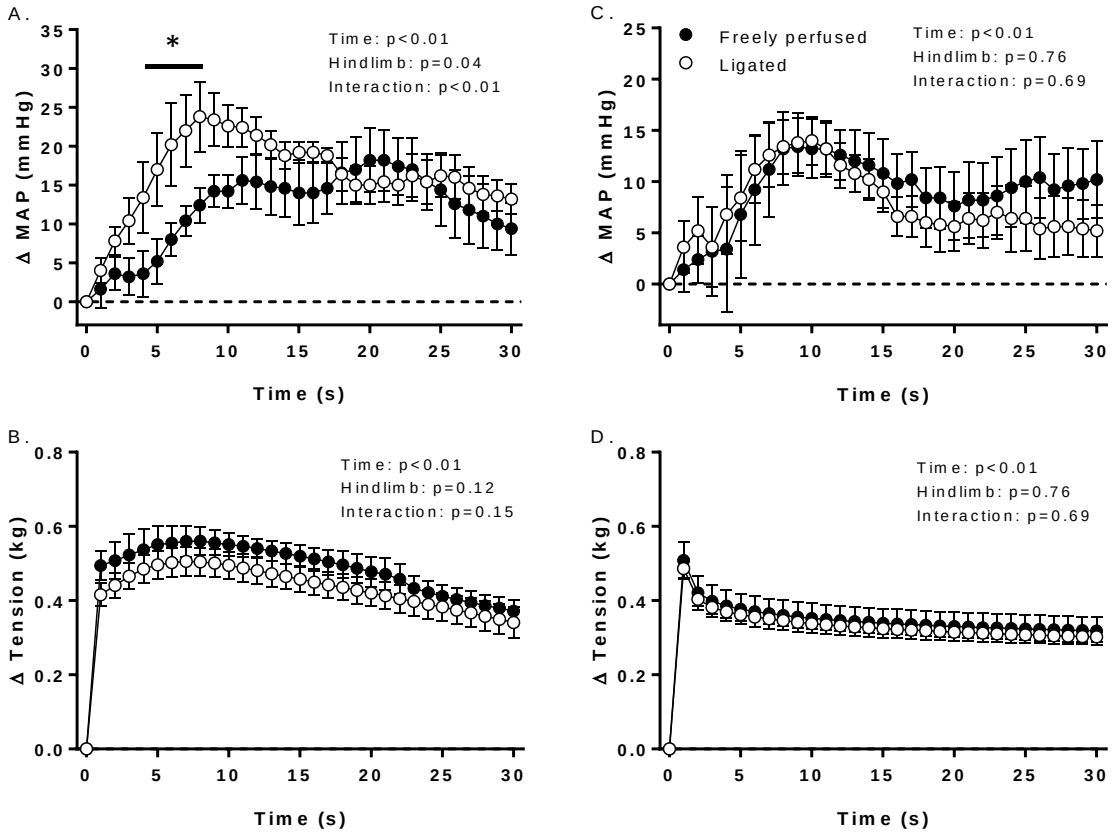

Figure 6: Pressor response to static contraction and static stretch of freely perfused and ligated hindlimbs

In five rats, the increase in mean arterial blood pressure (MAP) was greater during static triceps surae muscle contraction (panel A), but not static stretch (panel C), of the hindlimb in which the femoral artery was ligated compared to the contralateral hindlimb in which the femoral artery was freely perfused. The tension time courses during contraction (panel B) and stretch (panel D) were not different between hindlimbs. Data were compared with repeated measures two-way ANOVAs.

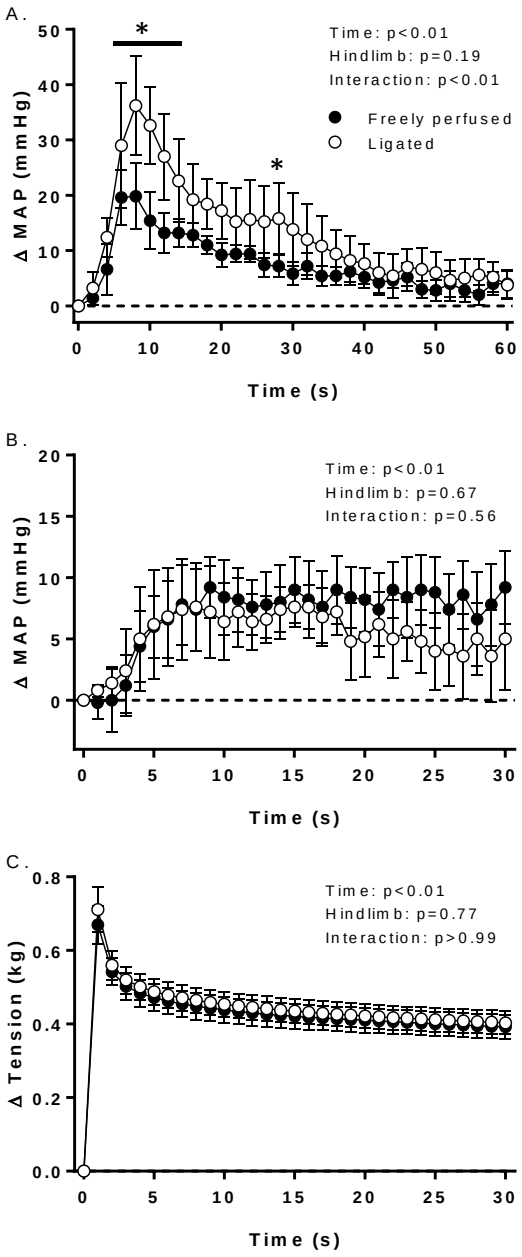

Figure 7: Pressor response to lactic acid injection and static stretch of freely perfused and ligated hindlimbs

In five rats, the increase in mean arterial pressure (MAP) was greater when lactic acid was injected into the arterial supply of the hindlimb in which a femoral artery was ligated compared

to the contralateral freely perfused hindlimb in which the femoral artery was freely perfused (panel A). In the same five rats, however, there was no difference in the response during static stretch of the hindlimbs (panel B). The tension time courses during stretch (panel C) were not different between hindlimbs. Data were compared with repeated measures two-way ANOVAs.

Protocol 1	**Control**	**Post-bradykinin**	**p-value**
Freely perfused (n=10)			
Baseline	421±16	445±14	0.03
Peak Δ	11±2	12±2	0.43
Ligated (n=10)			
Baseline	440±18	442±16	0.78
Peak Δ	10±3	14±3	0.09
Protocol 2	**Freely perfused**	**Ligated**	**p-value**
Stretch (n=10)			
Baseline	419±15	431±15	0.10
Peak Δ	12±2	11±2	0.67
Contraction (n=5)			
Baseline	422±29	433±23	0.22
Peak Δ	12±4	10±2	0.57
Stretch[#]			
Baseline	392±27	394±28	0.67
Peak Δ	11±2	13±2	0.43
Lactic acid injection (n=5)			
Baseline	425±16	423±15	0.35
Peak Δ	16±4	17±4	0.53
Stretch[^]			
Baseline	436±11	440±13	0.49
Peak Δ	6±1	6±2	0.65

Table 1: Baseline HR and peak increases (peak Δ) in HR (bpm) during experimental maneuvers

Data are mean±SEM and compared with paired student's t-test. The "#" symbol indicates that the stretches were performed in the same rats in which contraction was performed. The "^"

symbol indicates that the stretches were performed in the same rats in which the lactic acid injections were performed.

Chapter 4 - Discussion

The primary novel findings of this investigation were that: 1) the increase in blood pressure in response to the hindlimb arterial injection of bradykinin was greater in rats with a ligated femoral artery compared to rats with freely perfused femoral arteries, 2) the hindlimb arterial injection of bradykinin did not augment the increase in blood pressure or RSNA during triceps surae muscle stretch in either ligated or freely perfused rats, and 3) femoral artery ligation did not exaggerate the increase in blood pressure or RSNA during muscle stretch compared to the responses found in the freely perfused hindlimb of a different group of rats (protocol 1) or the contralateral freely perfused hindlimb within the same rat (protocol 2).

In protocol 1, we found that the increase in blood pressure was greater (i.e., the increase was prolonged) when bradykinin was injected into the arterial supply of a ligated hindlimb compared to the response evoked when it was injected into the arterial supply of a freely perfused hindlimb. Bradykinin has been shown to stimulate prostaglandin synthesis, which itself has been shown to influence thin fiber afferent responsiveness (18, 37). For example, Stebbins et al. (37) found that the increase in blood pressure that resulted from the hindlimb arterial injection of bradykinin in the cat was reduced by ~68% following inhibition of prostaglandin synthesis with indomethacin. Importantly, however, Pan et al. (29) found that bradykinin B2 receptor blockade practically abolished the bradykinin injection-induced increase in blood pressure. Taken together, those findings suggest that in a freely perfused hindlimb, the injection of bradykinin increases blood pressure via the stimulation of bradykinin B2 receptors and that the response is dependent, in large part, on PGE_2-induced bradykinin B2 receptor sensitization. More recently, Yamauchi et al. (46) found that the hindlimb arterial injection of PGE_2 increased blood pressure in ligated rats, whereas it decreased blood pressure in freely perfused rats.

Yamauchi et al. (46) also reported that the PGE_2 injection-induced increase in blood pressure in ligated rats was reduced by prior blockade of endoperoxide (EP, the receptor class stimulated by PGE_2) 3 and EP4 receptors and that femoral artery ligation exaggerated lumbar DRG EP4, but not EP3, receptor expression. Those findings, in conjunction with the finding that femoral artery ligation exaggerated lumbar DRG bradykinin B2 receptor expression (14), suggest that the exaggerated bradykinin injection-induced increase in blood pressure in the ligated rats in our study may have been due to greater bradykinin B2 receptor stimulation and PGE_2-induced bradykinin B2 receptor sensitization, as well as EP3 and/or EP4 receptor-mediated thin fiber afferent stimulation.

Multiple substances associated with muscle contraction, including bradykinin (12, 14, 19, 45), arachidonic acid and/or its cyclooxygenase byproducts (5, 20, 24, 32), lactic acid (33), and ATP (13), have been shown to play a role in sensitizing thin fiber muscle afferents. A particularly important sensitizing action of arachidonic acid and its byproducts, which includes PGE_2, on thin fiber muscle afferents has been reported in both humans (5, 20, 21) and animals (31, 32). In this study, we investigated the potential for bradykinin to sensitize skeletal muscle mechanoreceptors based on several recent important pieces of evidence that are summarized in the *Introduction*. It should be noted, however, that in addition to the evidence supporting the potential for bradykinin to sensitize muscle mechanoreceptors and the muscle mechanoreflex (6, 14), Mense and Meyer (19) found that exposure of the sensory endings of contraction-sensitive muscle afferents to a high dose of bradykinin (86 μg) desensitized the afferents' responsiveness to subsequent mechanical stimuli. Our present data, in contrast to our hypothesis, do not support the possibility of bradykinin-induced sensitization of the muscle mechanoreflex in either a ligated or freely perfused rat hindlimb.

We performed the post-bradykinin stretch in as close temporal proximity to its injection as possible (i.e., ~4 min), while also avoiding the increases in blood pressure that occurred in direct response to its injection. We cannot fully rule out the possibility that bradykinin sensitized muscle mechanoreceptors during the same time frame in which it directly activated the thin fiber muscle afferents. Our data do indicate, however, that any possible bradykinin-induced mechanoreceptor sensitization, if it does occur, does not persist beyond that time frame. Importantly, bradykinin-induced sensitization of P2X3 receptors (present investigation, 45), as well as prostaglandin-induced thin fiber afferent sensitization (32), does persist well beyond (i.e., at least ~3-20 min) the hindlimb arterial injection of those chemicals. Specifically, the pressor response to hindlimb arterial injection of α,β-methylene ATP was augmented above control values ~4 minutes (present investigation) and 20 minutes (45) following the hindlimb arterial injection of bradykinin. Moreover, Rotto et al. (32) found that group III afferent sensitization occurred during static contraction 3-6 min following hindlimb arterial injection of arachidonic acid.

In protocol 1, we did not find a difference in the pressor response evoked during triceps surae muscle stretch of a freely perfused and a ligated hindlimb when this comparison was made between different groups of rats. A review of the literature revealed that this comparison has only ever been made between different groups of rats and some studies reported that, compared to freely perfused rats, femoral artery ligation exaggerated the pressor response during muscle stretch (14-16, 46) whereas some studies reported that it did not (11, 42-44). We reasoned that a within-rat comparison between a freely perfused and ligated hindlimb would reduce variability that is often evident across different experimental preparations and would thus be the most appropriate experimental design to address this issue. In protocol 2, we found that the increases

in blood pressure, RSNA, and HR during triceps surae muscle stretch were not different when evoked from the hindlimb in which the femoral artery was previously ligated compared to the responses evoked from the contralateral freely perfused hindlimb. Moreover, we found no difference in the pressor response during stretch between hindlimbs in the same rats in which femoral artery ligation was found to exaggerate the pressor response during static hindlimb muscle contraction and lactic acid injection. Our finding that femoral artery ligation exaggerated the pressor response to static muscle contraction (41) and lactic acid injection (11, 15, 44) is consistent with previous reports. Because we found no difference in the pressor response during stretch between ligated and freely perfused hindlimbs, our data suggest that the enhanced contribution of mechano-gated channels to the exercise pressor reflex that was recently reported in ligated rats versus freely perfused rats (4) is attributable to an exaggerated acute metabolite-induced sensitization of muscle mechanoreceptors, rather than a chronic sensitization. As evident from the findings in protocol 1, however, bradykinin does not appear to play a role in this acute sensitization.

There are several limitations of our study. First, we stimulated muscle mechanoreceptors by stretching the triceps surae muscle whereas during most traditional forms of exercise/contractions skeletal muscle shortening and a simultaneous increase in intramuscular pressure likely underlies mechanoreceptor stimulation. Along this line, Hayes et al. (7) found in the cat that only 50% of the group III muscle afferents that responded to triceps surae muscle stretch also responded to muscle contraction. More recently, however, Stone et al. (39) found in freely perfused rats that 87%, and in ligated rats that 100%, of the group III muscle afferents that responded to stretch also responded muscle contraction. Thus, triceps surae muscle stretch in the rat constitutes a valuable experimental tool with which one can selectively activate the muscle

mechanoreflex to gain insights into the muscle mechanoreceptor stimulation that occurs during exercise. Second, atherosclerosis is a slowly developing process that results in a progressive narrowing of the arteries (27), whereas in our experiments the left femoral artery of the rats was completely occluded ~72 hours before the experiment. Nevertheless, chronic femoral artery ligation in the rat represents a model of simulated PAD which produces blood flow patterns at rest and during exercise that mimic closely those found in PAD patients at rest and during exercise (30, 48). We believe, therefore, that chronic femoral artery ligation in the rat constitutes a reliable model in which ischemia-induced alterations of the exercise pressor reflex may be investigated (40).

Perspectives and Significance

We found that the hindlimb arterial injection of bradykinin did not sensitize the increase in blood pressure, RSNA, or HR that occurred during selective activation of the muscle mechanoreflex via static triceps surae muscle stretch in either freely perfused or ligated rats (protocol 1). Moreover, we found that the increase in blood pressure during static triceps surae muscle stretch was not different between a ligated hindlimb and a freely perfused hindlimb when this comparison was made either between different groups of rats (protocol 1) or between hindlimbs within the same rat (protocol 2). The latter finding suggests that an exaggerated acute metabolite-induced sensitization of muscle mechanoreceptors underlies the exaggerated mechanically-sensitive component of the exercise pressor reflex that was found in rats with ligated femoral arteries (4). The former finding, however, casts doubt on the possibility that bradykinin plays a role in this sensitization. The rat model of chronic femoral artery ligation has been used as a model of simulated peripheral artery disease and, therefore, our findings may

have important implications for the exaggerated exercise pressor reflex that is found in PAD patients (25, 26).

Chapter 5 - References

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