Effect of chronic femoral artery ligation on skeletal muscle interstitial prostaglandin E_2 concentration in the rat: implications for mechanoreflex sensitization

by

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Abstract

Autonomic adjustments during exercise include a withdrawal of parasympathetic stimulation of the heart and an increase in the sympathetic stimulation of the heart and other organs/vascular beds. These adjustments are mediated, in part, by the exercise pressor reflex, a neural feedback mechanism activated by stimulation of group III and group IV afferents whose sensory endings are located within contracting skeletal muscle. Group III (mechanoreflex) and group IV (metaboreflex) afferents are primarily mechanically and metabolically sensitive, respectively. Cyclooxygenase (COX) products within skeletal muscles have the potential to sensitize the mechanical component of the exercise pressor reflex (i.e., mechanoreflex) in a variety of clinical populations including peripheral artery disease patients. For example, we found recently that COX inhibition reduced the pressor response evoked during hindlimb muscle stretch, a model of isolated mechanoreflex activation, in rats in which a femoral artery was ligated ~72 hours before the experiment (a model of simulated PAD in the rat). Conversely, we found no effect of COX inhibition on the mechanoreflex in normal, control rats. To investigate a potential mechanism of COX-mediated mechanoreflex sensitization in rats with ligated femoral arteries, we used the muscle microdialysis technique to test the hypothesis that femoral artery ligation in the rat increased resting skeletal muscle interstitial concentrations of the COX-metabolite prostaglandin E_2 (PGE_2). Contrary to our hypothesis, in 12 rats we found no difference in resting PGE_2 between control (i.e., freely perfused, 196±33 pg/ml) and the contralateral ligated (236±43 pg/ml, p=0.15) hindlimb. Moreover, we found that hindlimb muscle stretch increased PGE_2 concentration in freely perfused skeletal muscles (Δ PGE_2: 55±22 pg/ml, p=0.05) but not ligated (Δ PGE_2: 19±20 pg/ml, p=0.98) skeletal muscles. Collectively, the present results suggest that COX-mediated mechanoreflex sensitization in the ligated rat model of simulated PAD is not

attributable to elevated resting COX-metabolite levels or an exaggerated increase in COX-metabolite concentration during isolated mechanoreflex activation.

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Acknowledgements

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Dedication

I dedicate this work to the support system I have had throughout my academic career, especially my family and mentors. I specifically would like to thank my mother, Lori Felice and my sisters, Rylee and Kameron Felice.

Chapter 1 - Literature Review

Assessment of metabolites and other chemical compounds allow insight into normal homeostatic function and functional alterations that occur with progression of disease. Quantification of these metabolites allows investigators to tease apart the mechanisms underlying disease responses throughout the body. With developments in microdialysis, it is now possible to analyze a wide range of biological compounds across multiple species of experimental subjects. Microdialysis also offers the convenience of use in multiple tissue sites to further the understanding of systemic and local metabolite responses over time. This review will focus on the history and general concepts of microdialysis.

Microdialysis is an experimental technique in which a probe with a semipermeable membrane is placed within a tissue of interest (brain, skin, fat or muscle) and a solution, or 'perfusate,' is pumped through. This allows diffusion of metabolites and other biological compounds, which can be collected as a fluid termed, 'dialysate' (29, 56, 57). This fluid can be further analyzed and reflect concentrations from the site of interest. Bito et. al (1966) were the first to make use of a dialysis bag within the interstitial space (6). Microdialysis probes were then created by Delgado et. al (1972) and combined with perfusion of a solution via a dialysis bag carried to a specific site (13).

Early microdialysis investigations focused on the brain, specifically to measure neurotransmitter concentrations and the ability of pharmacological interventions to penetrate the blood brain barrier (13, 29, 56). This technique was further adapted for use in other tissues, mainly a decrease in probe size to expand applicable uses (29). An advantage of microdialysis is the ability to make effective comparisons using a limited sample size, and in some instances

perform multiple comparisons within the same animal (35). Microdialysis allows the measurement of dynamic changes over time while maintaining the surrounding physiological environment.

Microdialysis Technique

The earliest method of microdialysis employed a 'push pull' technique, where liquid was flushed directly through the tissue of interest in both directions to measure the contents of extracellular fluid (55). Following tissue disruption, if the proper balance of pushing and pulling was not achieved brain matter was removed during dialysate collection. As the technique progressed, a unidirectional flow pattern was adopted and microdialysis membranes were constructed in order to behave more similarly to a capillary.

A linear design is most common in skin and tissue investigation, where a membrane open on both ends is attached to tubing on each side (56, 65). In investigations of the brain, concentric probes are commonly used due to the stability and durability of the design. This probe type consists of a semi-permeable membrane with a single opening that forks in order to allow separate flow of solution into and out of the membrane. Loop probes, similar to the design of linear probes, side-by-side, and shunt probes are also commonly used. Probe types and design vary between investigations, and the benefits of each are dependent upon specific experimental conditions (29, 47).

Membrane length is critical in establishing how much of the probe is in direct contact with the tissue of interest. Microdialysis is primarily a diffusion-limited technique. Increased length allows a greater area of contact, but poses the challenge of greater insult to the site of interest (39, 47). There is also concern over the efficacy of probe placement as it becomes more

difficult to control for exact measurements at a specific target site. Membranes typically have a molecular weight (MW) cutoff, in which molecules exceeding the specified range cannot diffuse across the membrane or trace amounts recovered are negligible due to slow perfusion (58). A large molecular cut off is not believed to hinder the ability of a membrane to diffuse smaller molecules. Previous investigations have suggested that microdialysis membranes are most effective in collecting analytes one fifth to one sixth of the MW cutoff (29).

Following probe construction, the tubing attached to the membrane, or catheters, are attached to a perfusion pump in order to control the flow of perfusate. It is necessary to assess both flow into and out of the membrane, as several molecules, especially those of larger molecular weights, are sensitive to pressure changes (18). In instances where multiple probes have been placed, flow rate should be consistent across all probes. An increase in flow rate will increase the amount of dialysate collected, but diminish the relative recovery of an analyte across time (51). Slower flow rates increase level of equilibrium reached between probe and the surrounding environment, but decrease dialysate collection and add difficulty to analysis of analyte concentration.

Perfusate typically mimics normal physiological conditions with lower concentrations of endogenous compounds (ex. Saline, Ringer's solution) so as not to disrupt the environment of the tissue of interest. Preservative agents, such as antioxidants, can be added to the perfusate to aid in the recovery of specific analytes. These typically do not perfuse across the membrane, eliminating the possibility of affecting the surrounding tissue environment (51). Delivery of exogenous compounds is also possible, known as 'retrodialysis' (21). The diffusion of the perfusate containing a known concentration of an exogenous compound is measured. The

assumption is made that analytes diffuse at the same rate from the perfusate as those within the target site.

The Low-Flow- Rate method, demonstrated by Jacobson et. al, was one of the earliest descriptions of the relationship that existed between flow rate and membrane length. This investigation established the relationship between lower flow rates and an increase in yield of relative recovery. As flow rates decrease closer to zero, equilibrium most closely resembles that found within the tissue (22). This method yields low levels of dialysate and increases the length of the collection period due to low volume. It is also necessary to have instruments and equipment with increased sensitivity for proper analysis. This may lead to increased errors of analysis and degradation of biological components being investigated.

Human vs. Animal Model

Investigations centered on microdialysis have become common in both human and animal models. In humans, the periphery, including skin and muscle, have been the main focus of dialysate collection. Studies concerning the brain have been conducted in individuals with brain injuries specifically due to the difficult nature of probe placement, longevity of experimental protocols and the awareness of human subjects (4, 10, 28). Assessment of brain tissue, specifically diagnosis of various neurological disorders, can be completed using microdialysis. Efficacy and bioavailability of pharmacological interventions has also been explored (35). Investigations within skeletal muscle and subcutaneous skin assessments have become possible (15, 23, 29, 35-38). MacLean et. al were the first investigators to apply the microdialysis technique to dynamic exercise in humans. Using knee extensor exercise, their

investigation assessed glucose and lactate concentration and the ability of microdialysis probes to continue recovery at rest and during activity (20, 38).

Significant research has occurred in the animal model of microdialysis experiments. Multiple species have been investigated in the brain, skin, muscle, fat and several other tissue sites. Similar blood brain barrier transport properties and the ability to successfully predict target site concentrations in humans based on rat data have been noted in several investigations (5, 16, 31).

Interstitial Space

Measurement of interstitial fluid at the site of the muscle, allows for data representing the unbound concentration of muscle derived compounds available to act on receptors specifically (15, 30, 36, 37). The environment of the muscle most accurately represents activity of the afferents, as levels of metabolites and synthetization of other proteins and enzymes may be watered down if measured from a site of systemic activity such as the jugular vein.

While much of microdialysis has been focused on chronic pain, receptor expression and metabolite activity that affects the pain response also combine to produce the exercise pressor reflex (EPR) (26, 52). In the diseased state, metabolic by products of arachidonic acid, are upregulated and act to sensitize group III and IV afferents (7, 30). Increased receptors expression, increased binding affinity, and a lower threshold of activation are potential systemic responses to chronic inflammation. Inflammation is associated with multiple forms of cardiovascular disease, including peripheral artery disease (PAD).

Peripheral Artery Disease

PAD is defined by the buildup of atherosclerotic plaque within the vasculature, typically supplying the lower limb. This leads to the ability to maintain perfusion of skeletal muscle at rest

but an inability to increase blood flow during activity (17). Changes in the vasculature supplying the lower limb occur in an effort to increase blood supply to the tissue. While density of micro vessels may increase in PAD, it is not sufficient to offset the lack of perfusion during activity (2). These adaptations can improve exercise tolerance, even if oxygen delivery is not increased. Increases in collateral flow are able to continue delivery of flow further downstream, mitigating the effects of ischemia at rest. Treatment options during PAD, including exercise and some pharmacological interventions, may act to 'induce' the expansion of vasculature to improve blood flow and oxygen delivery (17, 34, 63).

Multiple animal models have been used in an effort to further our understanding of PAD (17, 27). A rat model of simulated PAD in which the femoral artery is tied off (ligated), mimics blood flow patterns of those found in human subjects where perfusion of resting muscle experiences modest to no effect with ligation (2, 9). The exercise pressor reflex is also augmented in this rat model of PAD, but only during contraction of the musculature of the ischemic limb (17, 54). Both mechanically-mediated (mechanoreceptor) and metabolically-mediated (metaboreceptor) afferents are affected. Over activation of the mechanoreflex is thought to contribute to the underlying mechanism of the augmented EPR in multiple forms of cardiovascular disease (43, 44, 45).

Substantial build-up of inflammatory markers occurs within 3 days of limb ischemia in the ligated rat model of PAD (8). Pathways associated with inflammation may act to sensitize group III afferents, or increase signaling from mechanically activated receptors. The cyclooxygenase (COX) pathway has been linked to EPR dysfunction in PAD (44). When considering how this interaction occurs, an increase in COX metabolites may mediate the chronic sensitization of the mechanoreflex evident in PAD. It is also possible that over

expression of receptors associated with this pathway occur or further adaptations in second messenger systems. Increasing the understanding of metabolite production and changes across time may over insight into the functional alterations of the EPR in PAD.

Conclusion

Microdialysis is a cost effective, minimally invasive experimental technique that can be used in a variety of experimental protocols. The ability to assess concentrations of multiple biological compounds allows investigators to quantify changes in concentration in response to activity, disease, and pharmacological interventions. Continued improvements in probe constructions and design have expanded the applicability of this technique. Future directions may lead to better understanding of maladaptation in disease at the systemic and local level, allowing investigators to further tease apart the mechanistic underpinnings of physiological dysfunction.

Chapter 2 - Introduction

Autonomic adjustments at the onset exercise include decreased parasympathetic activation of the heart, with an increase in sympathetic stimulation of the heart and other organs/ vascular beds. These are mediated in part by the exercise pressor reflex, a neural feedback mechanism activated by the stimulation of group III and group IV afferents, whose sensory endings are located within contracting skeletal muscle. These classes of afferents are primarily mechanically and metabolically sensitive, respectively (1, 11, 25, 40). Activation of the exercise pressor reflex leads to an increase in perfusion of the working muscle. Increased metabolic demand mediated by muscle ischemia led to long-standing interest in the metabolic component of this feedback mechanism (i.e., metaboreflex). Recent investigations into the mechanically mediated arm of the reflex (i.e., mechanoreflex) have found that alterations in group III afferent signaling augment reflex responses found in several forms of cardiovascular disease (32, 41, 43-45, 53).

The arachidonic acid cascade, primarily cyclooxygenase 1 and 2 (COX-1 and COX-2) is a key mediator in activation of both the metabo- and mechanoreflex. COX activity produces thromboxanes and prostaglandins, leading to accumulation of these metabolites in skeletal muscle during activity (50). In healthy human and animal subjects, a link between COX metabolites and responsiveness of the mechanoreflex has been demonstrated during exercise (12, 19, 42). Mechanoreflex over activation specifically underlies the augmented reflex responses to activity found in hypertension (32), heart failure (41, 43), and peripheral artery disease (44). Metabolically mediated sensitization of the mechanoreflex has been linked directly to the COX pathway in heart failure and PAD. COX inhibition in both conditions attenuates the reflex

response to mechanoreflex activation, suggesting a correlation between a buildup of COX metabolites, such as PGE_2, and an increased sensitization of group III afferents (14, 41-44).

Microdialysis is an experimental technique in which a probe containing a semi-permeable membrane is inserted directly into the muscle. Fluid termed 'dialysate' is collected and can be analyzed to determine metabolite concentration within the interstitium (57). Metabolites in this space act directly on the sensory endings of afferents located within contracting skeletal muscle (42). This technique measures dynamic changes over time, allowing assessment of basal levels of various muscle derived compounds and shifts in concentration in response to activity. Recently our lab demonstrated a significant increase in the production of PGE_2 in healthy rats during isolated mechanoreflex activation via passive tendon stretch using microdialysis. In this same investigation, COX inhibition via indomethacin (a non-selective COX inhibitor) did not affect the pressor response to primarily mechanically mediated reflex activation (49). These findings are in agreement with Muller et. al in which systemic inhibition of COX via ketorolac, a non-selective COX inhibitor, decreased reflex responses to mechanoreceptor activation in human subjects with peripheral artery disease but not healthy counterparts (44).

While COX mediated sensitization plays a role in the augmented reflex response to activity in humans with PAD, it is difficult to determine the location of this sensitization due to systemic drug delivery. It is also difficult to determine the role of the mechanoreflex specifically, as it is impossible to definitively isolate activation of group III afferents in human subjects. Passive limb movement with human subjects introduces possible error during investigations due to the effect of perceived effort and mental state, activation of unintended muscle groups during activity, and increased inter-subject variability.

The use of an animal model to study the pressor response to activity, allows investigators to activate the mechanoreflex in the absence of contraction-induced metabolites. Ligation of the rat hindlimb is a model of simulated PAD, in which the femoral artery is 'tied off' or occluded ~72 hours prior to an experiment. This animal model of PAD mimics the increased sympathetic nerve responses and augmented exercise pressor reflex found in human subjects (27, 54). Functional alterations in the vasculature also lead to a reduction in blood flow where perfusion is able to meet the demands of skeletal muscle at rest but not during activity (48, 62-64).

In recent experiments, our laboratory found that indomethacin (a non-selective COX inhibitor) attenuated the pressor response in ligated rats during hindlimb muscle stretch, a model of isolated mechanoreflex activation in the absence of contraction induced metabolites. This data suggests COX mediated-mechanoreflex sensitization occurs with chronic femoral artery ligation in the rat, similar to results found in human PAD counterparts who saw an attenuation of the EPR during plantar flexion exercise with COX inhibition. (44).

While the COX pathway plays a role in over activation of the mechanoreflex in PAD, where in this pathway sensitization occurs has yet to be identified. A potential mechanism is a build-up of COX metabolites. In the current investigation, we sought to determine if the augmented mechanoreflex found in PAD occurred due to chronic sensitization from a difference in resting levels of PGE_2 in a ligated rat model of simulated PAD when compared to freely perfused hindlimbs. We hypothesized that the concentration of PGE_2 in muscle microdialysate would be elevated at rest in ligated hindlimbs. We also investigated the effect of ligation on production of PGE_2 within the rat hindlimb during mechanoreflex activation via dynamic passive tendon stretch. Based on our previous findings, we hypothesized that an exaggerated increase in

PGE_2 would occur in ligated rat hindlimbs with isolated mechanoreflex activation using dynamic passive tendon stretch.

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Chapter 3 - Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. *In vivo* experimental techniques were performed on young adult (~12-15 weeks) male Sprague- Dawley rats (n=15, average body weight: 459±11 g, Charles River Laboratories). Rats were housed two per cage in temperature (maintained at ~22 °C) and light (12-12-hour light-dark cycle running 7 AM to 7 PM) controlled facilities. Standard rat chow and water was provided *ad libitum*. At the end of these experiments, the decerebrated rats (see below) were killed by an intravenous injection of saturated (>3 mg/kg) potassium chloride.

Surgical Procedure: Twelve of the 15 rats used in this investigation had their left femoral artery ligated (referred to as "ligated rats" from this point forward for simplicity) 72-96 hours before the experimental protocol. Rats were briefly anesthetized with ~3% isoflurane (balance O_2). The left femoral artery was then surgically exposed and ligated tightly with 5-0 silk suture ~3-5 mm distal to the inguinal ligament. The incision was then closed and meloxicam (1-2 mg/kg sc) was administered as an analgesic.

On the day of the experiment, rats were anesthetized. 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, Holliston, MA) with a 2% isoflurane-balance O_2 mixture until decerebration was complete (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, measurement of arterial blood pressure (Physiological Pressure

Transducer, AD Instruments), and sampling of arterial blood gases. For all experiments, the skin covering the left hindlimb was cut and reflected to expose triceps surae (gastrocnemius, soleus and plantaris) muscles. In 12 rats, this procedure was completed on both hindlimbs. For stretch experiments (n=10), a string was attached to the Achilles tendon and the calcaneus was severed. The triceps surae muscle was then linked to a force transducer (Grass FT03) which was attached to a rack and pinion.

Following completion of the initial surgical procedures, all rats were placed in a Kopf stereotaxic frame. Dexamethasone (0.2 mg i.v.) was administered to minimize swelling of the brainstem. A precollicular decerebration was performed and all neural tissue rostral to the superior colliculus was removed. Once decerebration was complete, anesthesia was terminated and the lungs were ventilated with room air. Decerebration was performed because anesthesia dampens the exercise pressor reflex in the rat. Arterial blood gases and pH were measured with a blood gas analyzer (ABL80 Flex, Radiometer) and maintained within normal physiologic parameters ($PaCO_2$: 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 a heat lamp.

Probe Construction Microdialysis probes were constructed by gluing both ends of a pre-measured membrane (Spectra/Por®, 13-kDa molecular cutoff) to PE tubing, with one end attached to a syringe hub. In protocol 1, membrane lengths used included 1 cm, 1.75 cm, and 2 cm. Following results of these experiments, all other probes were constructed using a membrane measuring 1.75cm. On the day of each experiment, membranes were flushed with ethanol to

‘activate’ the membrane. Activation of membranes is required, as they are purchased containing a filling liquid to ensure structural integrity and protect the membrane. This liquid is alcohol soluble. A second flush of distilled water or perfusate solution removes traces of ethanol, and ensures the probe is ready for placement.

Protocol 1- Three microdialysis probes with semi-permeable membranes were placed and attached to a perfusion pump. All membranes were completely submerged in a lactate solution in a dish and allowed sufficient time to reach equilibrium. Concentration of the lactate solution was known, and measurements were taken throughout the duration of the protocol to ensure that concentration was similar across time. The solution was stirred using a mixing plate at a low speed to keep the solution consistent throughout. Flow rates ranging from 2.5- 40 µl/min were used, with lactate measurements taken immediately as dialysate was produced. Measurements were taken in duplicate using a lactate analyzer for each measured flow rate. Averages of these values were calculated and analyzed.

Muscle microdialysis Microdialysis experiments were performed on 15 rats. Probes were attached to a syringe filled with saline placed within a microdialysis pump to allow for constant infusion and controlled flow rate. Following decerebration, in 12 of 15 rats used, four microdialysis probes were placed within the right and left triceps surae muscle. A 20-gauge needle was used to puncture the muscle and guide placement of the probe. In each hindlimb, two probes were placed within the medial gastrocnemius, one in the lateral gastrocnemius, and one within the soleus muscle (see Fig.1). This was chosen in order to sample each muscle fiber type

and represent total activity of the hindlimb. Sufficient recovery time (~120 minutes) was allowed following probe placement before fluid collection began.

Protocol 2- Following the previous investigation, membranes of 1.75 cm were placed within the triceps surae muscles of the left hindlimb as described above (n=3). Dialysate from all probes was pooled into a single collected sample for immediate analysis. Lactate measurements were recorded using the same analysis technique from the previous protocol. Measurements at flow rates ranging from 2.5-40 µl/min were recorded in duplicate.

Protocol 3- Following establishment of a protocol *in vivo,* 1.75 cm length probes were placed in the same location (see Fig. 1), and constantly infused with saline at 5 μl/min. In rats with a ligated model of simulated PAD, probes were placed within both the freely perfused and ligated hindlimb, to allow within animal comparisons between conditions. Collection tubes were placed within a cold block to slow the degradation of the biological components of the dialysate. A 90-minute recovery period was used prior to muscle microdialysate fluid collection, following no significant difference in resting PGE_2 values assessed at 60 minutes post probe placement and 120 minutes post probe placement. We utilized a 90-minute waiting period following these findings to maintain consistency throughout each investigation. Following collection of dialysate at rest, baseline tension was set to 80-100g of tension. Fluid was collected for four minutes to analyze pre-stretch baseline. In random order, freely perfused and contralateral ligated hindlimbs were dynamically stretched for four minutes.

A skilled investigator from our laboratory, rhythmically turned a rack and pinion at 1 Hz achieving similar tension with each stretch for 4 minutes. This length of stretch was chosen to ensure that enough dialysate was produced for analysis of samples. A ~10-minute recovery period was used between maneuvers. Following this recovery period, the stretch protocol was repeated with the opposite hindlimb. Dialysate tubes were stored in a cold block during collection. In order to account for dead space and ensure dialysate collected represented the composition of the interstitial space during stretch, fluid collection did not begin until ~2-minutes following the initiation of the stretch protocol.

Our laboratory had previously conducted control experiments using Evans Blue dye in which we measured the time for the dye to travel through the muscle, into the probe, and emerge as dialysate for collection. During stretch experiments, fluid was also collected for four minutes following completion of the stretch protocol, as this dialysate was within the muscle at the time of interest. Following collection, all samples were immediately stored at – 80 °C. Analysis of fluid was completed using a commercially available PGE_2 ELISA kit (Arbor Assay) using the low-sample volume yield protocol. To construct the standard curve, samples were ran in triplicate. Dialysate collected at rest was ran in duplicate. The coefficient of determination (r^2) of the standard curve across all assays was 1 (range 0.99-1). Average coefficient of variation of duplicate samples across all assays was 5% (range 3-9%).

Data Analysis Data was collected with a PowerLab and LabChart data acquisition system (AD Instruments). Blood pressure, muscle tension and ECG were measured and recorded in real time. Mean arterial pressure (MAP) and heart rate (HR) were calculated and displayed in real time, and recorded for offline analysis. The Δ tension time index (Δ TTI) was calculated for each

maneuver by integrating the area under the tension signal during the stretch protocol and subtracting the integrated area during the baseline period. The 30-second baseline TTI was multiplied by the appropriate factor to equate it to the duration of stretch. All values are expressed as mean ± SE. Data was analyzed using paired student's t-test and repeated-measures ANOVAs with Holm-Sidak post hoc tests. Statistical significance was defined as $p \leq 0.05$.

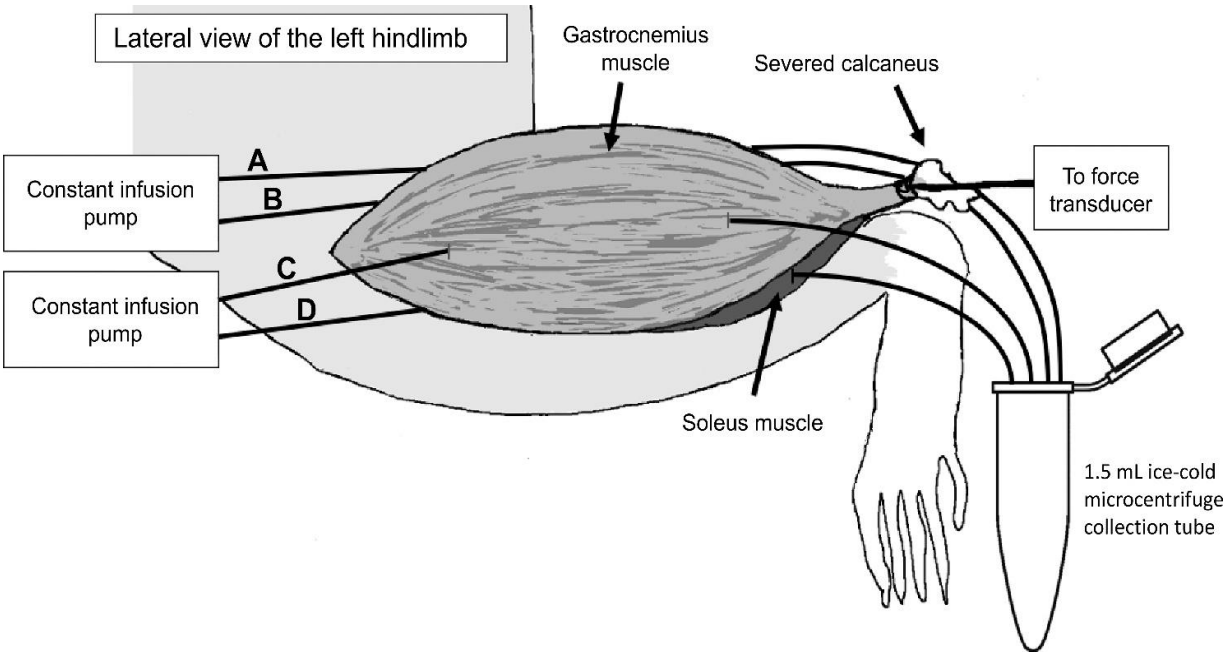


Figure 1: Probe placement during muscle microdialysis

Schematic illustrating placement of microdialysis probes during each experiment following surgical separation of the rat hindlimb. Probes A and B were placed in the medial gastrocnemius. Probe C was placed in the lateral gastrocnemius and probe D was placed in the soleus. Dialysate from all four probes was pooled into a single sample for immediate analysis during lactate experiments. All dialysate was pooled in a single collection tube for storage until use for analysis during experiments measuring PGE_2 concentration, at rest and during mechanoreflex activation via passive tendon stretch.

Chapter 4 - Results

Protocol 1: *Effect of probe length and flow rate on Lactate*

Probes measuring 1cm, 1.75 cm and 2 cm were constructed in order to measure the effects of probe length and flow rate on diffusion of lactate. As probe length increased, relative recovery of lactate from the lactate bath also increased. An inverse relationship became evident as flow rate increased, the recovery of lactate decreased. (See Fig.2) This relationship was true across all membrane lengths. Similar recovery was measured between 1.75 cm and 2 cm length membranes across multiple flow rates (Fig. 2). This protocol served as a control to assess the ability of our method to measure the concentration of an analyte of interest. A flow rate of 5 µl/min was chosen for future microdialysis investigations, as it yielded the greatest concentration of lactate (1.7 mmol) while still allowing for the greatest collection of dialysate fluid. Slower flow rates allow greater diffusion to occur, but decrease the amount of dialysate collected. Small amounts of fluid produced make quantification difficult and poses the challenge of degradation of the analytes of interest (29)

Protocol 2: *Lactate measurements in vivo*

Based on the results of the previous experiments, we selected a membrane length of 1.75 cm for use in measuring lactate concentration within the hindlimb. This membrane length was chosen to ensure the membrane was completely encapsulated within the hindlimb. Experiments were performed in three rats. Lactate has been investigated extensively with the microdialysis technique, during activity and at rest. (37) With an increase in flow rate, there was a reduction in

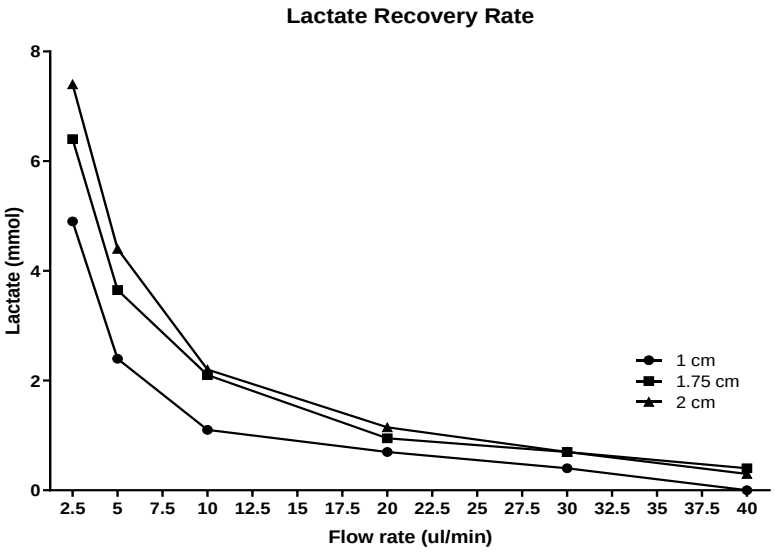
lactate recovered from the hindlimb (see Fig. 3). Results of these experiments confirmed the ability to measure metabolite concentration within the rat hindlimb.

Protocol 3: *PGE_2 measurements*

In 12 rats, experiments were conducted to compare resting concentrations of PGE_2 between freely perfused and contralateral ligated hindlimbs. There was no significant difference in resting muscle microdialysate fluid PGE_2 concentration between freely perfused (196±33 pg/ml) and the contralateral hindlimb that had been previously ligated (236±43 pg/ml, p=0.15)). (See Fig 4). Baseline MAP (freely perfused: 76±9, ligated: 78±91, p=0.09) and baseline heart rate (freely perfused: 443±41, ligated: 457±39, p=0.22) were not significantly different between freely perfused and ligated rats during dialysate collection.

In 10 of these 12 rats, we performed a dynamic stretch protocol to isolate mechanoreflex activation within freely perfused and contralateral ligated hindlimbs. In this subset of rats, changes in PGE_2 concentration from rest to stretch were quantified. Following the stretch protocol, a significant increase in PGE_2 above baseline levels occurred in the freely perfused hindlimb (p=0.05) (Fig. 5), while there was no stretch-induced increase in PGE_2 in the ligated hindlimb (p=0.98) (Fig. 5). The ΔTTI of the stretch maneuvers was not different between hindlimbs (p=0.35). Baseline HR (freely perfused: 444±41, ligated: 461±41, p=0.22) and baseline MAP (freely perfused: 72±8, ligated: 74±9, p=0.09) were not significantly different between freely perfused and ligated rats during stretch.

A.)



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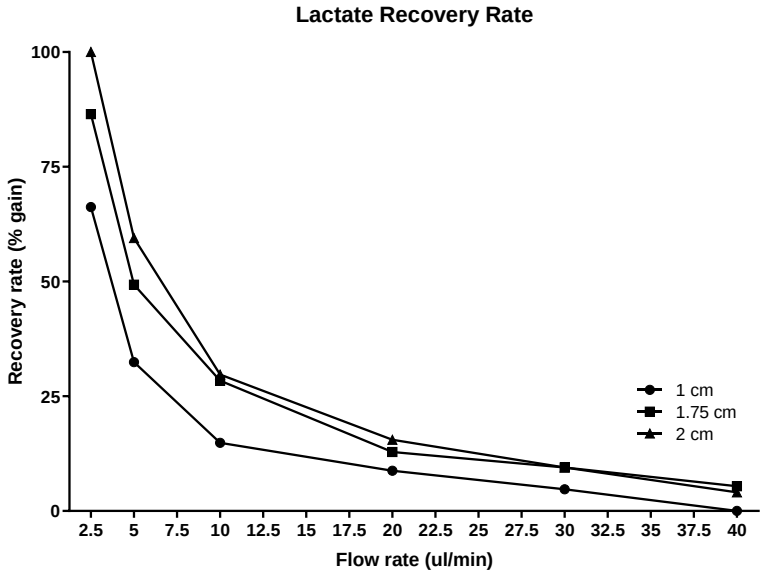

Figure 2: Effect of membrane length and flow rate on lactate recovery

A and B as membrane length (cm) increased, concentration (mmol) and relative recovery (% gain) of lactate within microdialysate fluid also increased. An inverse relationship became evident across all membrane lengths, as an increase in flow rate (µl/min) negatively affected lactate readings. Lactate measurements were taken in duplicate and mean values are reported.

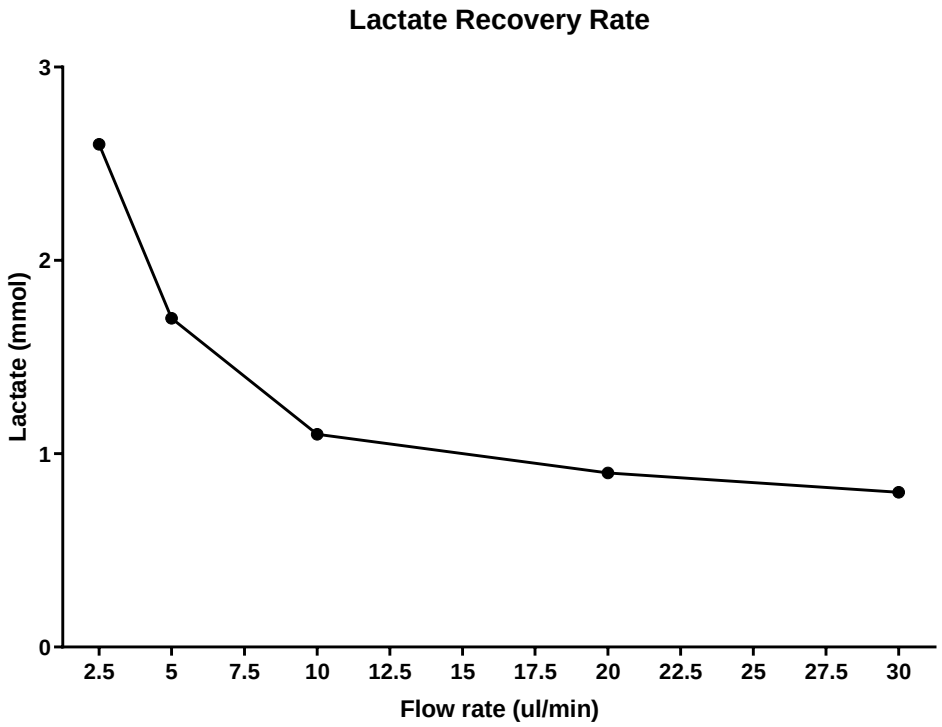

Figure 3: Relative recovery of lactate *in vivo*

As flow rate increased, the amount of lactate (mmol) recovered in muscle microdialysate fluid from the triceps surae muscle decreased. Flow rates tested varied from 2.5-40 µl/min. At 40 µl/min, lactate readings were below the detectable range of the lactate analyzer used. Measurements were taken in duplicate with mean values expressed.

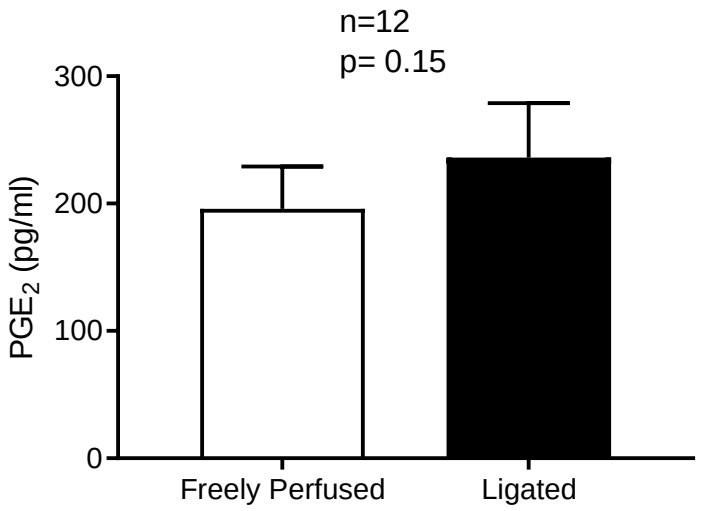

Figure 4: Resting concentration of PGE_2 in freely perfused and ligated hindlimbs

No significant difference (n=12) was found between resting concentration of PGE_2 within microdialysate fluid collected from freely perfused (196±33 pg/ml) and ligated (236±43 pg/ml) hindlimbs (p=0.15). Values are mean ±SE. Data were analyzed with a paired student's t-test. Statistical significance was accepted at $p \leq 0.05$.

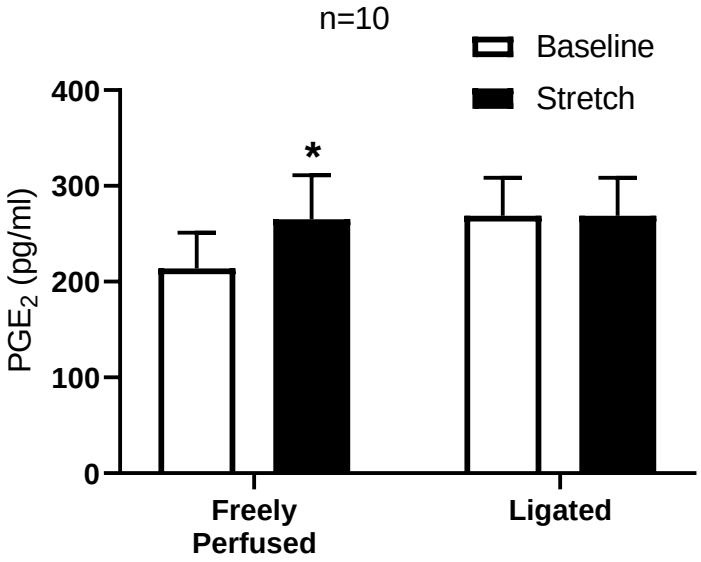

Figure 5: Measured increase of PGE_2 concentration with mechanoreflex activation

Dynamic stretch increased the concentrations of PGE_2 within muscle microdialysate fluid above resting levels (n=10) in freely perfused hindlimbs ($p=0.05$) but not ligated hindlimbs ($p=0.98$). Muscle tension produced during the stretch maneuver was not different between hindlimbs. Values are mean ± SE. Data were analyzed with a 2-way RM ANOVA with Holm-Sidak post hoc tests. Statistical significance was accepted at $p \leq 0.05$.

Chapter 5 - Discussion

Novel findings of our investigation include: 1.) Resting levels of interstitial PGE_2 concentration are not elevated in the ligated rat hindlimb compared to freely perfused 'healthy' counterparts and 2.) Increases in PGE_2 during mechanoreflex activation via passive dynamic hindlimb stretch are not exaggerated in a ligated rat model of simulated PAD.

In protocol 1 and 2, we established a muscle microdialysis protocol for use in the ligated rat model of simulated PAD. Microdialysis investigations are commonly used in the brain, primarily analysis of pharmacological interventions (4-6, 13, 16, 29, 51). We utilized this technique to assess the environment acting on group III and group IV afferents to gain further insight on the mechanistic adaptations that occur in response to disease.

This investigation began by assessing the ability to construct microdialysis probes and determine the relationship that existed between membrane length and flow rate. A curvilinear relationship became evident across all membrane lengths (1 cm, 1.75 cm, and 2 cm). As flow rate increased the concentration of lactate recovered decreased. Following the ability to successfully measure lactate concentrations using a standard lactate solution with a known concentration, we transitioned to using microdialysis probes *in vivo*.

During this protocol, we assessed the ability of our probes to diffuse a known metabolite within skeletal muscle. Lactate has successfully been investigated with the microdialysis technique within a variety of tissues (35, 46). A relationship between flow rate and membrane length was further confirmed. Slower flow rates also yielded greater concentrations of measured lactate within muscle dialysate. A curvilinear relationship was evident. Following successful measurements of a metabolite across time and distinguishing different concentrations, we progressed to measuring PGE_2 concentration in the hindlimb of freely perfused and ligated rats.

Microdialysis has also been used to assess concentrations of prostaglandins in human subjects (24). COX-mediated sensitization of the mechanoreflex has been demonstrated in various forms of cardiovascular disease (41, 43, 44, 50). Our laboratory found that COX inhibition with indomethacin (a non-selective COX inhibitor), significantly reduces the reflex response to passive tendon stretch in a ligated rat model of simulated PAD. Reduction of the pressor response confirmed that COX-mediated sensitization of the mechanoreflex occurs with ligation. Synthesis of PGE_2 as it relates to baseline levels within skeletal muscle has previously been suggested as the rate-limiting step of COX inhibition.

We sought to determine if elevated concentrations of PGE_2 within the interstitial space of the rat hindlimb acts to sensitize the mechanoreflex with ligation. We have shown that resting levels of PGE_2 are not significantly different between freely perfused and contralaterally ligated hindlimbs. While these findings refute our initial hypothesis, these are in line with Leal et. al, who measured resting levels of TXB_2 (another COX metabolite) and found no significant difference in resting levels between freely perfused and ligated hindlimbs (33). Paired with our findings, we conclude that an increase in resting levels of COX metabolites is not the mechanism underlying chronic sensitization of the mechanoreflex in a rat model of simulated PAD.

Mechanical stimulation of skeletal muscle creates an increase in PGE_2 efflux leading to accumulation of this metabolite, and this efflux may play a key role in activation of reflex responses to activity (24). Previous findings from our laboratory utilizing passive tendon stretch in freely perfused animals, demonstrated a significant increase above baseline levels of PGE_2 produced during stretch. Rollins et al, demonstrated that COX inhibition via indomethacin (a non-selective COX inhibitor) had no effect on reflex responses to mechanoreflex activation in freely perfused, ‘healthy’ rats (49). With the knowledge that COX mediated sensitization

occurred during ligation, we measured if there was an exaggerated increase of PGE_2 during passive tendon stretch in the ligated hindlimb compared to freely perfused counterparts.

Following a ~4 min passive tendon stretch protocol to isolate mechanoreflex activation in the absence of contraction induced metabolites, a significant increase in the concentration of PGE_2 occurred in freely perfused but not ligated hindlimbs. This was an important confirmation of our previous findings in freely perfused rats, but also demonstrated there is not an exaggerated increase in PGE_2 concentration during isolated mechanoreflex activation following ligation of the femoral artery. No difference in metabolite concentration without the aid of drug intervention suggests that an over activity in production is not the rate limiting step of chronic sensitization.

While resting levels of PGE_2 and synthesis during stretch of PGE_2 are not different between freely perfused and ligated rats, there are multiple sites within the COX pathway that may serve as the mediator to chronic sensitization of the mechanoreflex. Receptors expressed within group III afferents demonstrate binding affinity to several metabolites including PGE_2, thromboxanes and leukotrienes (45). Leal et. al demonstrated, in the same study previously mentioned, that blockade of the TP receptor (part of the COX pathway, acted on by thromboxanes) with daltroban, in both health and disease, attenuated the cardiovascular reflex responses to stretch (33). Yamauchi et. al demonstrated that blockade of Endoperoxide 4 (EP4) receptors (acted upon directly by PGE_2) in PAD was able to attenuate the reflex response during contraction. This investigation showed no effect of EP4 blockade in health, suggesting a role in the transition to disease (59). While both classes of receptors are involved in producing the exercise pressor reflex during health, EP4 receptors play a larger role in augmenting the mechanoreflex in PAD. Adaptations within skeletal muscle may include over-expression of

multiple receptor types or an increased binding affinity. It is also possible that sensitization occurs due to a lower threshold of activation of each receptor.

Group III afferents demonstrate increased sympathetic out-pouring to mechanical deformation and sensitivity to metabolite build up at the onset of activity (19, 25, 44, 50). While resting levels of PGE_2 concentration were not elevated in the ligated hindlimb, it is possible that chronic sensitization occurs due to accumulation of another metabolite. Certain receptors may also display increased sensitivity to metabolites depending on the progression and severity of disease.

Changes in pathway function of the EPR have been noted in varying stages of heart failure (HF), also characterized by mechanoreflex over activation. (41, 43). The COX pathway has also been implicated as playing a direct role in chronic sensitization of the mechanoreflex during HF. Morales et al. recently demonstrated a reduction in mechanoreflex responsiveness with the use of a selective COX-2 inhibitor in HF. (43). Over expression of COX-2 but not COX-1 has been found in skeletal muscle of patients with HF (3). Paired with the findings from our present investigation, differentiating the enzymatic activity and protein expression of COX 1 and 2 may be beneficial in PAD. Targeted treatments may prove more effective when considering the COX pathway completely.

Limitations An important limitation of our study is simulated PAD with femoral artery ligation. In a clinical setting, PAD occurs overtime, while the model used in this investigation occurs in ~72 hours. Previous investigations from our laboratory (27) have shown similar increases in the augmented reflex response, as those measured in humans with PAD (27, 54). Adaptations in collateral flow in the rat model of PAD mimic those found in human subjects and

the ability to maintain muscle perfusion at rest but not with the onset of activity is also similar (17, 48, 60-62).

It is also possible that the length of our stretch protocol impacted measurements of PGE_2 synthesis. A standard 30 sec protocol to activate the mechanoreflex could not be used, due to utilizing the microdialysis technique. In order to collect enough fluid for analysis, a longer stretch protocol was required. Dilution of recovered metabolites seems unlikely. If exaggerated production of PGE_2 occurred during the initial phase of stretch, it is likely that this would extend throughout the remaining portion of the stretch protocol.

Perspectives and Significance Our study suggests that while cyclooxygenase products may play a role in sensitization of group III afferents in the ligated rat model of PAD, this is not due to elevated resting levels of PGE_2 or an exaggerated increase in PGE_2 concentration during isolated mechanoreflex activation. Assessment of receptors tied to activation of group III afferents and quantification of protein expression associated with the COX pathway may serve as future directions in gaining further insight into the mechanisms underlying the augmented mechanoreflex in PAD.

References

1. **Alam M, Smirk FH.** Observation in man upon a blood pressure raising reflex arising from the voluntary muscles. *J Physio*l 89: 372-383, 1937.
2. **Angersbach D, Jukna JJ, Nicholson CD, Ochlich P, Wilke R**. The effect of short-term and long-term femoral artery ligation on rat calf muscle oxygen tension, blood flow, metabolism and function. *Int J Microcirc Clin Exp* 7: 15-30, 1988.
3. **Antunes-Correa LM, Nobre ST,, Groehs RV, Alves MJ, Fernandes T, Couto GK, Rondon MU, Oliveira P, Lima M, Mathias W, Brum PC, Mady C, Almeida DR, Rossoni LV, Oliveira EM, Middlekauff HR, Negrao CCE**. Molecular basis for the improvement in muscle metaboreflex and mechanoreflex control in exercise-trained humans with chronic heart failure. *Am J Physiol Heart and Circ Physiol* 307: H1655-H1666, 2014.
4. **Azeredi F, Dallas CT, Derendorf H**. Role of microdialysis in pharmacokinetics and pharmacodynamics: current status and future directions. *Clin Pharmacokinet* 53: 205-212, 2014.
5. **Ball K, Bouzom F, Scherrmann J, Walther B, Declèves X**. Comparing translational population-PBPK modelling of brain microdialysis with bottom-up prediction of brain-to-plasma distribution in rat and human. *Biopharmaceutics & Drug Disposition* 35: 485-499, 2014.
6. **Bito L, Davson H, Levin EM, Murray M, Snider N**. The concentration of free amino acids and other electrolytes in cerebrospinal fluid, in vivo dialysate of brain and blood plasma of the dog. *J Neurochem* 13: 1057-1067, 1966.

7. **Botting RM, Botting JH**. Pathogenesis and mechanisms of inflammation and pain. *Clin Drug Invest* 19: 1-7, 2000.
8. **Brevetti G, Giuglisno G, Brevetti L, Hiatt WR**. Inflammation in peripheral artery disease. *Circulation* 122: 1862-1875, 2010.
9. **Brevetti LS, Paek R, Brady SE, Hoffman JI, Sakar R, Messina LM**. Exercise-induced hyperemia unmasks regional blood flow deficit in experimental hindlimb ischemia. J Surg Res 98: 21-26, 2001.
10. **Chen JW, Rogers SL, Gombart ZJ, Adler DE, Cecil S**. Implementation of cerebral microdialysis at a community- based hospital: a 5-year retrospective analysis. *Surg Neurol Int* 3: 57, 2012.
11. **Coote JH, Hilton SM, Perez-Gonzalez JF**. The reflex nature of the pressor response to muscular exercise. *J Physiol* 215: 789-804, 1971.
12. **Cui J, Moradkhan R, Mascarenhas V, Momen A, Sinoway LI**. Cyclooxygenase inhibition attenuates sympathetic responses to muscle stretch in humans. *Am J Physiol Heart Circ Physiol* 294: H2693-H2700, 2008.
13. **Delgado JMR, DeFeudis FV, Roth RH, Ryugo DK, Mitruka BM**. Dialytrode for long term intracerebral perfusion in awake monkeys. *Arch Int Pharmacodyn Ther* 198: 9-21, 1972.
14. **Drew RC, Muller MD, Blaha CA, Mast JL, Herr MD, Stocker SD, Sinoway L**I. Aspirin augments carotid-cardiac baroreflex sensitivity during muscle mechanoreflex and metaboreflex activation in humans. *J Appl Physiol* 115: 1183-1190, 2013.
15. **Green S, Bulow H, Saltin B**. Microdialysis and the measurement of muscle interstitial K+ during rest and exercise in humans. *J Appl Physiol* 87: 460-464, 1999.

16. **Gusstafson S, Eriksson J, Syvöanen S, Eriksson O, Hammarlund-Udenaes M, Antoni G**. Combined PET and microdialysis for in vivo estimation of drug blood-brain barrier transport and brain unbound concentrations. *J NeuroImage* 155: 177-186, 2017.
17. **Haas TL, Lloyd OG, Yang HT, Terjung Rl**. Exercise training and peripheral artery disease. *Compr Physiol* 2: 2933-3017, 2012.
18. **Hammarlund-Udenaes M.** Microdialysis as an important technique in systems pharmacology- a historical and methodological review. *AAPS J* 19: 1294-1303, 2017.
19. **Hayes SG, Kindig, Kaufman MP**. Cyclooxygenase blockade attenuates responses of group III and group IV afferents to dynamic exercise in cats. *Am J Physiol Heart Circ Physiol* 290: H2239-H2246, 2006.
20. **Hickner RC**. Applications of microdialysis in studies of exercise. *Exercise and sports science reviews* 28: 117-122, 2000.
21. **Höcht C, Opezzo JAW, Taira CA**. Applicability of reverse microdialysis in pharmacological and toxicological studies. *J Pharmacol Toxicol Methods* 55: 3-15, 2007.
22. **Jacobson I, Sandberg M, Hamberger A**. Mass transfer in brain dialysis devices-a new method for estimation of extracellular amino acids concentration. *J Neurosci Methods* 15: 263-268, 1985.
23. **Kalant H**. A microdialysis procedure for extraction and isolation of corticosteroids from peripheral blood plasma. *The Biochemical Journal* 59: 99-103, 1958.
24. **Karamouzis M, Langberg H, Skovgaard D, Böulow J, KJÆr M**. In situ microdialysis of intramuscular prostaglandin and thromboxane in contracting skeletal muscle in humans. *Acta Physiol Scand* 171: 71-76, 2001.

25. **Kaufman, M, Hayes S**. The Exercise Pressor Reflex. *Clinical Autonomic Research*, 12: 429-439, 2002.

26. **Kaufman MP, Iwamoto GA, Longhurst JC, Mitchell JH**. Effects of capsaicin and bradykinin on afferent fibers with endings in skeletal muscle. *Circ Research* 50: 133-139, 1981.

27. **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* 314: H246-H254, 2018.

28. **Kennedy RT**. Emerging trends in in vivo neurochemical monitoring by microdialysis, *Curr Opin Chem Biol* 17: 860-867, 2013.

29. **Kho CM, Ab Rahim SKE, Ahmad ZA, Abdullah NS**. A review on microdialysis calibration methods: the theory and current related efforts. *Mol Neurobiol* 54: 3506-3527, 2017.

30. **Kuehl F, Egan RW**. Prostaglandins, arachidonic acid and inflammation. *Science* 210: 978-984, 1980.

31. **Langer O, Karch R, Muller U, Dobrozemsky G, Abrahim A. Zeitlinger M, Lackner E, Brunner M**. Combined PET and microdialysis for in vivo assessment of intracellular drug pharmacokinetics in humans. *J Nucl Med* 46: 1835-1841, 2005.

32. **Leal AK, Williams MA, Garry MG, Mitchell JH, Smith SA**. Evidence for functional alterations in the skeletal muscle mechanoreflex and metaboreflex in hypertensive rats. *Am J Physiol Heart Circ Physiol* 295: H1429-H1438, 2008.

33. **Leal A, McCord J, Tsuchimochi H, Kaufman M**. Blockade of the TP receptor attenuates the exercise pressor reflex in decerebrated rats with chronic femoral artery occlusion. *Am J Physiol, 301:* H2140, 2011

34. **Lloyd PG, Yang HT, Terjung RL**. Arteriogenesis and angiogenesis in rat ischemic hindlimb: role of nitric oxide. Am J Physiol Heart Circ Physiol 281: H2528-H2538, 2001. .

35. **Lönnroth, P**. Microdialysis--a new and promising method in clinical medicine. *Journal of Internal Medicine,* 230: 363-4, 1991.

36. **Lott ME, Hogeman CS, Vickery L, Krunselman AR, Sinoway LI, MacLean DA**. Effects of dynamic exercise on mean blood velocity and muscle interstitial metabolite responses in humans. *Am J Physiol Heart Circ Physiol* 281: H1734-H1741, 2001.

37. **Lott ME, Sinoway LI**. What has microdialysis shown us about the milieu within exercising skeletal muscle?. *Exerc Sport Sci Rev* 32: 69-74, 2004.

38. **MacLean DA, Bangsbo J, Saltin B**. Muscle interstitial glucose and lactate levels during dynamic exercise in humans determined by microdialysis. *J Appl Physio* 87: 1483-1490, 1999.

39. **Mathy FX, Denet AR, Vroman B, Clarys P, Barel A, Verbeeck RK, Préat V**. In vivo tolerance of assessment of skin after insertion of subcutaneous and cutaneous microdialysis probes in the rat. *Skin Pharmacol Appl Skin Physiol* 16: 18-27, 2003.

40. **McCloskey DI, Mitchell JH**. Reflex cardiovascular and respiratory responses originating in exercising muscle. J Physiol 224: 173-186, 1972.

41. **Middlekauff HR, Chiu J, Hamilton MA, Fonarow GC, MacLellan WR, Hage A, Moriguchi J, Patel J**. Cyclooxygenase products sensitize muscle mechanoreceptors in humans with heart failure. *Am J Physiol Heart Circ Physiol* 294: H1956-H1962, 2008.

42. **Middlekauff HR, Chiu J**. Cyclooxygenase products sensitize muscle mechanoreceptors in healthy humans. *Am J Physiol Heart Circ Physiol* 287: H1944-H1949, 2004.
43. **Morales A, Gao W, Lu J, Xing J, Li J**. Muscle cyclo-oxygenase-2 pathway contributes to the exaggerated muscle mechanoreflex in rats with congestive heart failure. *Exp Physiol* 97: 943-954, 2012.
44. **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* 309: H523-H528, 2015.
45. **Murphy MN, Mizuno M, Mitchell JH, Smith SA**. Cardiovascular regulation by skeletal muscle reflexes in health and disease. *Am J Physiol Heart Circ Physiol* 301: H1191-H1204, 2011.
46. **Okuda C, Sawa T, Harada M, Murakami T, Matsuda T, Tanaka Y**. Lactate in rat skeletal muscle after hemorrhage measured by microdialysis probe calibrated in situ. *Am J Physiol* 26: E1035-E1039, 1992.
47. **Plock N, Kloft C**. Microdialysis—theoretical background and recent implementation in applied life-sciences. *European journal of pharmaceutical sciences* 25: 1-24, 2005.
48. **Prior BM, Lloyd PG, Ren J, Li H, Yang HT, Laughlin MH, Terjung RL**. Time course of changes of collateral blood flow and isolated vessel size and gene expression after femoral artery occlusion in rats. *Am J Physiol Heart Circ Physiol* 287: H2434-H2447, 2004.
49. **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 decerabrate rats. *Am J Physiol Regul Integr Comp Physiol* 317: R369-R378, 2019.

50. **Rotto DM, Schultz HD, Longhurst JC, Kaufman MP**. Sensitization of group III muscle afferents to static contraction by arachidonic acid. *J Appl Physiol* 68: 861-867, 1990.
51. **Shippenberg TS, Thompson AC**. Overview of microdialysis. *Curr Protoc Neurosci* 7: 1-15, 2008.
52. **Simone DA, Marchettini P, Graziella C, Ochoa JL**. Identification of muscle afferents subserving sensation of deep pain in humans. *J Neurophysiol* 72: 883-889, 1994.
53. **Stone AJ, Kaufman MP**. The exercise pressor reflex and peripheral artery disease. *Autonomic Neuroscience: Basic and Clinical* 188: 69-73, 2015.
54. **Tsuchimochi H, McCord JL, Hayes SG, Koba SG, Kaufman MP**. Chronic femoral artery occlusion augments exercise pressor reflex in decerebrated rats. *Am J Physiol Heart Circ Physiol* 229: H106-H113, 2010.
55. **Ungerstedt U, Hallström A**. In vivo microdialysis-a new approach to the analysis of neurotransmitters in the brain. *Life sciences* 41: 861-864, 1987.
56. **Ungerstedt U, Pycock C**. Functional correlates of dopamine neurotransmission. *Bull Schweiz Akad Med Wiss* 1278: 1-13, 1974.
57. **Ungerstedt U**. Microdialysis- principles and applications for studies in animals and man. *J of Internal Medicine* 230: 365-373, 1991.
58. **Wang X, Stenken JA**. Microdialysis sampling membrane performance during in vitro macromolecule collection. *Anal Chem* 78: 6026-6034, 2006.

59. **Yamauchi K, Kim JS, Stone AJ, Ruiz-Velasco V, Kaufman MP**. Endoperoxide 4 receptors play a role in evoking the exercise pressor reflex in rats with simulated peripheral artery disease. *J Physiol* 591: 2949-2962, 2013.

60. **Yang HT, Deschenes MR, Ogilvie RW, Terjung RL**. Basic fibroblast growth factor increases collateral blood flow in rats with femoral artery ligation. *Circulation Research* 79: 62-69, 1996.

61. **Yang HT, Feng Y, Allen A, Protter A, Terjung R**. Efficacy and specificity of bFGF increased collateral flow in experimental peripheral arterial insufficiency. *Am J Physiol Heart Circ Physiol* 278; H1966-H1973, 200.

62. **Yang HT, Feng Y**. bFGF increases collateral blood flow in aged rats with femoral artery ligation. *Am J Physiol Heart Circ Physiol* 278: H85-H93, 2000.

63. **Yang HT, Ogilvie RW, Terjung RL**. Heparin increases exercise-induced collateral blood flow in rats with femoral artery ligation. *Circulation Research* 76: 448-456, 1995.

64. **Yang HT, Terjung R**. Angiotensin- converting enzyme inhibition increases collateral-dependent muscle blood flow. *J Appl Physiol* 75: 452-457, 1993.

65. **Zuo, H, Meng Y, and Davies M**. Monitoring transdermal delivery of nicotine using in vivo microdialysis sampling. *Current Separations* 15: 63-66, 1996.