ENDOTHELIAL AND SMOOTH MUSCLE-DEPENDENT VASCULAR REACTIVITY IN IMMATURE ARTERIALIZED COLLATERAL CAPILLARIES

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PROJECT INFORMATION

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ABSTRACT

Endothelial and Smooth Muscle-Dependent Vascular Reactivity In Immature Arterialized Collateral Capillaries

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Peripheral arterial occlusive disease (PAOD) occurs due to the build up of atherosclerotic plaque and reduces blood flow to cause chronic ischemia. Patients with PAOD may experience intermittent claudication, or the pain in limb skeletal muscles due to a decease in blood flow. Collateral arteries can act as a natural bypass and improve blood flow to hypoxic tissue by creating an alternate route for blood to flow, but not all patients with PAOD have pre-existing collateral networks. Animal studies indicate that tissues without pre-existing collateral networks can form de novo collaterals from capillaries following occlusion of a feed artery. Unfortunately, theses de novo collaterals, termed arterialized collateral capillaries (ACCs) lack functional vasodilation at day-7 following feed artery occlusion. To induce the formation of ACCs, we ligated the lateral feed artery in the spinotrapzeius muscle in Balb/c mice. We evaluated the potential mechanism of impaired functional vasodilation in immature arterialized collateral capillaries (7 days following occlusion) by measuring endothelial-dependent vasodilation to bradykinin and endothelial-independent vasodilation to isoproterenol and sodium nitroprusside. Vasodilation to both the endothelial-dependent and endothelial-independent vasodilators was impaired in the immature ACCs as compared to the terminal arterioles on the unoperated sham side. Similar responses to the endothelial cell and smooth muscle cell-dependent vasodilations suggest that impaired functional vasodialtion is due to impaired vascular smooth muscle cell function, which is consistent with our preivous research. We speculate that the SMCs of the ACCs are immature and may still be remodeling, rearranging, or modulating phenotype in the newly formed collaterals. Determining factors to induce mature arterialized collateral capillaries in patients with PAOD lacking pre-existing collateral netoworks could reduce ischemia and improve prognosis.

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"I believe there are no questions that science can't answer about a physical universe."
—Stephen Hawkings

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INTRODUCTION

Clinical Relevance

Peripheral arterial occlusive disease (PAOD) is a serious disease that increases one's chance of coronary heart disease. It is caused by atherosclerosis that results in the luminal stenosis of arteries and reduced blood flow to limb skeletal muscles. Atherosclerosis is the buildup of plaque in arteries and can lead to chronic ischemia, the decrease in blood flow to tissue, and impede function in microcirculation (**Figure 1**) [20]. Ischemia due to PAOD often leads to cramping and pain in skeletal limb muscles due to the insufficient delivery of oxygen and nutrients. Intermittent claudication, or hypoxic pain during locomotion, is the most common symptom of PAOD and is intensified during exercise or stress.

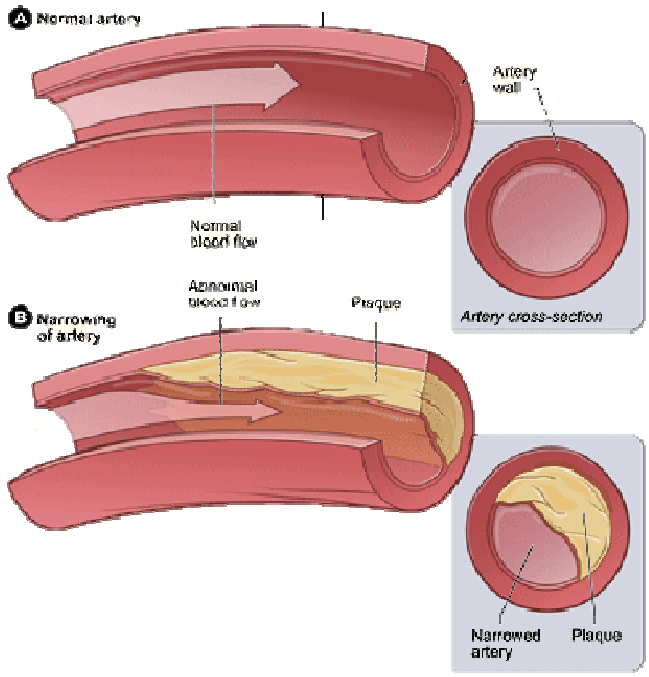

Figure 1: Atherosclerotic plaque in an artery. Schematic image comparing a normal artery and an artery with atherosclerotic plaque buildup [22].

Treatments for PAOD seek to manage symptoms of the disease, reduce atherosclerotic plaque, and restore blood flow to ischemic regions. Common treatments include lifestyle modifications and pharmaceuticals that aim to reduce the intermittent claudication. More serious cases of PAOD may require stenting or bypass surgery. Bypass surgery involves using an autologous saphenous vein to bypass the occluded artery and restore blood flow. Yet not all patients are eligible for bypass surgery due to pre-existing conditions or the lack of a robust vein to serve as a conduit. Therefore, developing alternate treatments for PAOD is necessary to benefit all patients. Natural bypass vessels can improve prognosis of patients with PAOD by providing blood flow around the occluded artery. Collateral vessels provide blood flow to ischemic areas by bypassing the diseased vessel, and the growth of collateral arteries can be a life-saving occurrence after an arterial occlusion [18].

Previous Work

Pre-existing collateral networks improve the prognosis of patients with PAOD, but not all patients have robust collaterals and need an alternative for redirecting blood flow [14]. Animal studies indicate that genetic background strongly influences the number and remodeling of pre-existing collateral arteries, suggesting that patients who lack robust collaterals may either lack pre-existing collaterals or exhibit impaired arteriogenesis. The mechanisms of arteriogenesis are being studied in different models in order to enhance the process of collateral formation in patients with PAOD [7]. Studies on the microcirculation in different mice strains show that it is possible to evaluate two types microcirculation networks in Balb/c and C57 mice.

Balb/c mice have a dendritic, unconnected arteriole tree structure whereas C57s have an arcade structure with many arteriole-to-arteriole connections. Accordingly, with fewer, if any, pre-existing collaterals, collateral vessels in Balb/c mice form by remodeling at the capillary

level after an occlusion [14]. Collateral vessels provide increased blood flow to ischemic areas of tissue, therefore it is important to understand the formation of arterialized collateral capillaries in ischemic models lacking pre-existing collaterals.

Arterialized collateral capillaries (ACCs) can form in animals that lack pre-existing collaterals when an occlusion is present, and allow blood to flow around the occlusion through a robust capillary [14]. In order for sufficient blood to flow to the ischemic region, the ACCs must exhibit functional vasodilation, which is impaired at certain time points in animal models and can prevent sufficient blood flow during strenuous activities [9].

Balb/c mice showed no functional vasodilation in arterialized capillaries 7 days post ligation, suggesting the ACCs were immature at this time point [3]. Achytalcholine (EC-dependent), sodium nitroprusside (SMC-dependent), and norepinephrine (SMC-dependent) reagents were applied to the spinotrapezius muscle of Balb/c mice, the results from which suggested that the impairment of vasodilation at 7 days post ligation is due to smooth muscle cell impairment [9]. It is important to study the impairment of collateral vessels in different models to understand how recovery from an occlusion can be accelerated.

Endothelial-Dependent Dilation

Bradykinin (BK), isoproterenol (ISO), and sodium nitroprusside (SNP) were applied to the spinotrapezius muscle of Balb/c mice in a physiological salt solution seven days post ligation to understand the vascular reactivity in arterialized collateral capillaries. Endothelial dependent (bradykinin) and endothelial independent (isoproterenol and sodium nitroprusside) reagents were utilized to test the different pathways that cause smooth muscle cells to relax and provide functional vasodilation of arterioles. Endothelial cells cause smooth muscle cells to relax through different pathways [16].

Bradykinin is an endothelial-dependent agonist that interacts with G protein coupled receptors (GPCR) at the endothelial cell surface [6]. The binding of BK to the GPCR activates phospholipase C (PLC) and causes an increase in cytoplasmic calcium and diacylglycerol (DAG) levels [10,17]. The surplus of intracellular calcium binds to calmodulin (Cam) and phosphorylates eNOS to promote the synthesis of NO [19]. NO is released into smooth muscle cells where it binds to soluble guanylyl cyclase receptors (sGC) [7, 4]. The activation of sGC increases cGMP production, activating protein kinase G (PKG) that phosphorylates myosin light chain kinase. Phosphorylation inhibits MLCK and decreases the interaction between actin and myosin, causing relaxation and vasodilation [2]. BK can increase blood flow and improve myocardial metabolism through vasodilation of microvessels [11].

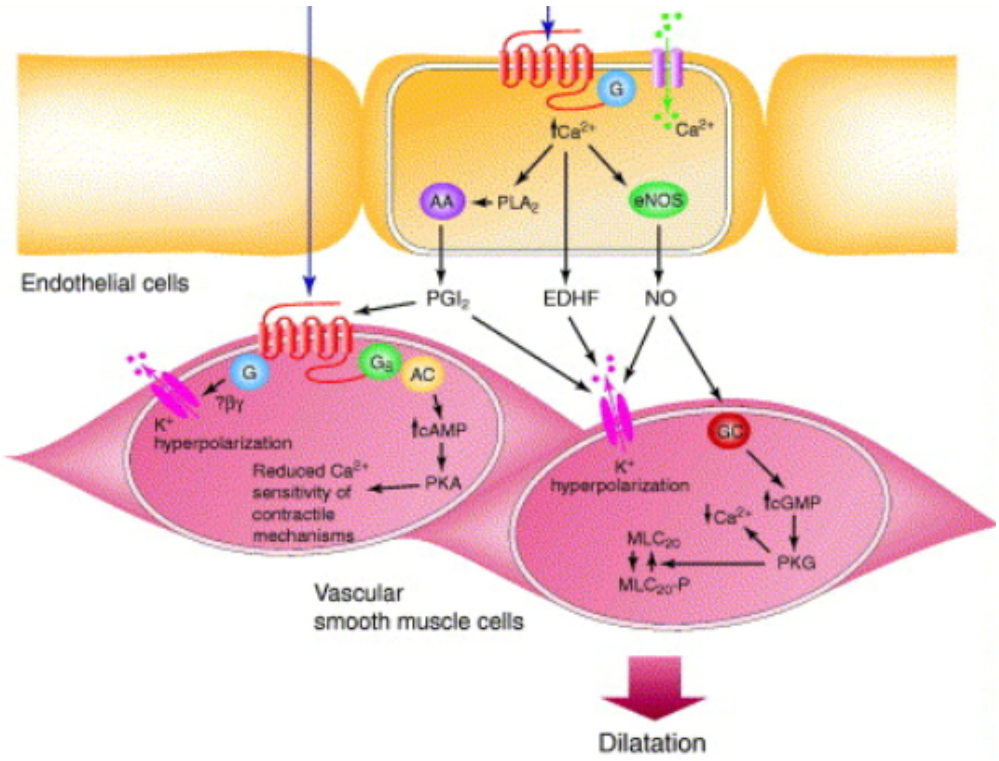

Figure 2: Endothelial-dependent vasodilation. Bradykinin acts through the GPCR on endothelial cells in microvasculature to cause dilation of smooth muscle cells [15].

Previous research examined the impairment of vasodilation in vasculature using acetylcholine (Ach), another endothelial-dependent agonist that acts through a different signal transduction pathway. Ach targets the GPCR and activates the protein kinase A (PKA) in the GPCR cascade [14, 8]. There was no endothelial impairment of vasodilation in immature arterialized capillaries when stimulated with Ach [9], beyond the smooth muscle cell impairment that was observed.

Smooth muscle cells in microvasculature are able to relax independently of endothelial-derived factors. Smooth muscle cell-dependent dilators, such as epinephrine, acts through beta-adrenergic receptors to relax the smooth muscle cells in microvasculature. Beta-adrenergic receptors are GPCRs, that when stimulated by their agonist, cause the dissociation of the G_{α} subunit and the activation of adenylyl cyclase. Adenylyl cyclase (AC) catalyzes the conversion of ATP into cyclic-AMP (cAMP) and the cAMP phospohorylates cAMP-dependent PKA, which phosphorylates MLCK. Phosphorylation of the MLCK causes a decrease in activity, hyperpolarization and relaxation of the smooth muscle cells [12]. Isoproterenol is a nonselective beta-adrenergic agonist that stimulates beta-receptors and causes relaxation of arterioles by directly targeting smooth muscle cells.

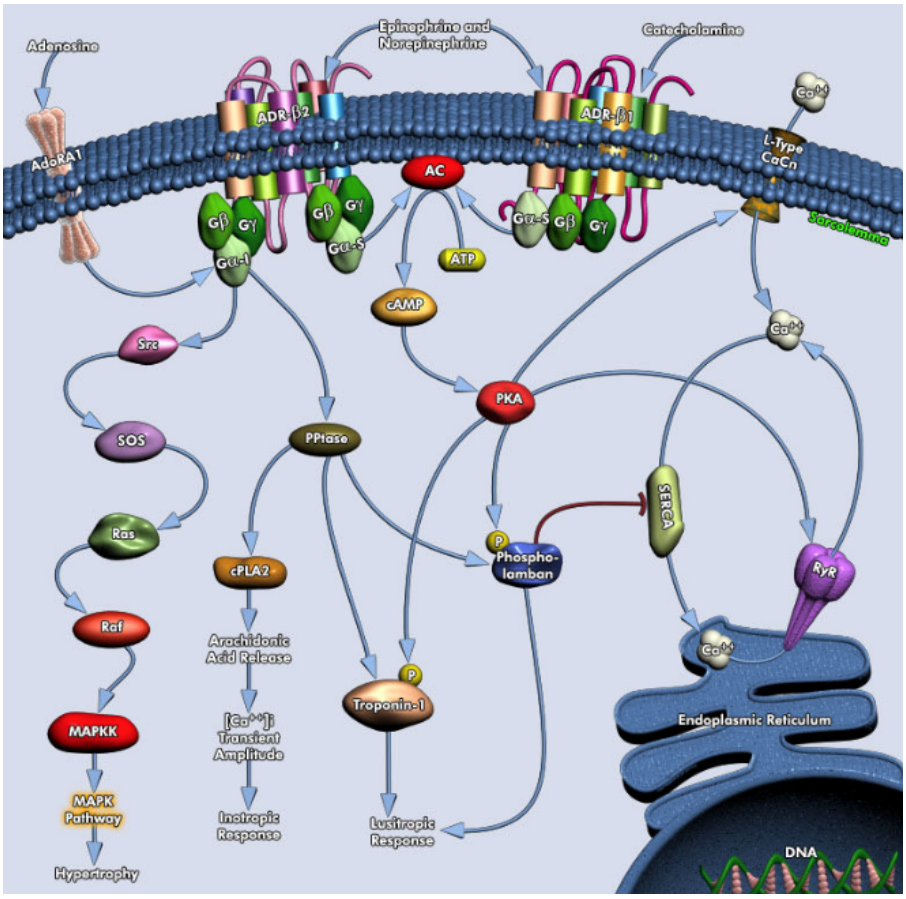

Figure 3: Beta-adrenergic signaling cascade. Endothelial-independent dilators cause smooth muscle cell relaxation through the dissociation of the G_{α} subunit [21].

Sodium nitroprusside is a smooth muscle cell-dependent vasodilator that bypasses the endothelial activation of the GPCR. SNP is converted into NO that diffuses directly into the SMCs and causes vasodilation through an increase in cGMP and the phosphorylation of the myosin light chain kinase. Sodium nitroprusside acted as a control in this study, as previous studies have analyzed the effect of SNP on vasodilation of immature arterialized capillaries in Balb/c mice and saw impaired vascular reactivity through the NO pathway [9].

These reagents were chosen to target specific cell types and extend the work of previous studies that utilized Ach, SNP and NE to examine impaired vascular reactivity in arterialized capillaries.

Specific Aims

It is hypothesized that the impaired vasodilation in immature arterialized collateral capillaries is due to smooth muscle cell impairment. If the SMC is impaired, we expect to see similar responses in the ACCs to BK and ISO on the ligated side as well as similar responses between BK and Ach and ISO and SNP [9]. Arterialized collateral capillary formation can benefit patients that lack pre-existing collateral networks and to maximize the benefit of collaterals, the ACCs must be capable of regulating blood flow and exhibiting functional vasodilation.

Specific Aim 1: To assess the reactivity of terminal arterioles in unoperated mice and identify the lowest concentration of BK and ISO necessary to achieve maximum vasodilation.

Specific Aim 2: To test the hypothesis that smooth muscle cell-dependent vasodilation is impaired in arterialized collateral capillaries 7 days post ligation of the lateral feed artery in the spinotrapezius muscle.

METHODS

Animal Care

Male Balb/c mice were used in the procedures in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of California Polytechnic State University, San Luis Obispo. Mice were housed in the University Vivarium and were monitored daily for food and water levels.

Lateral Feed Artery Ligation

Ligations were performed to occlude the lateral feed artery and induce the formation of ACCs in the spinotrapzeius muscle. Buprenorphine analgesic (0.075 mg kg^{-1}) was subcutaneously administered after the mouse was anesthetized with 5% isoflurane in oxygen flowing at 3-5 l⋅min^{-1}. The mouse was then transferred to the heat pad on the surgical stage where a nose cone administered ~1-3% isoflurane flowing at 0.5-1.0 l⋅min^{-1}. The heat pad was set to 35° C for the duration of the procedure and the temperature was monitored with a rectal probe. The hair on the dorsal region of the mouse was removed prior to the surgery with clippers and depilatory cream, and veterinary ointment was applied to the corneas to prevent corneal desiccation.

Using aseptic technique, a small incision was made above the cranial, lateral edge of the spinotrapezius muscle, where the fat pad overlays the muscle. The fat pad, cranial and superficial to the spinotrapezius, was blunt dissected to expose the lateral edge; the site was frequently irrigated with phosphate buffered saline (PBS) to prevent desiccation. The lateral feed artery was isolated and ligated with single strands from 6-0 silk suture (**Figure 2**). The initial incision was closed with 7-0 polypropylene suture. Sham-side surgeries were performed

to induce similar trauma to the area without ligating the artery. Immediately following surgery, buprenorphine analgesic was administered subcutaneously (0.075 $mg \cdot kg^{-1}$). The operated mouse was returned to the vivarium and given oral buprenorphine (0.01 $mg \cdot mL^{-1}$) for two days following surgery.

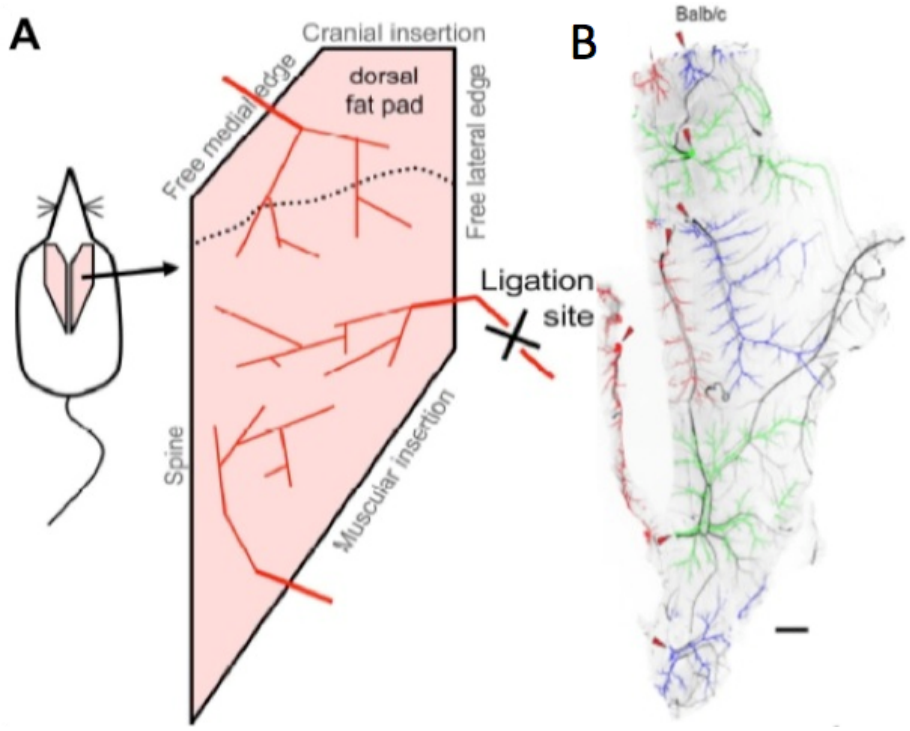

Figure 4: Spinotrapezius artery feed ligation. A. Schematic of spinotrapezius muscle. B. Balb/c vasculature lacking collaterals [14].

Vascular Reactivity

Seven days post ligation, the spinotrapezius muscle was exposed to vasoactive agents on both the ligated and sham side to examine endothelial-dependent and endothelial-independent vasodilation of arterialized collateral capillaries (ACCs).

The mouse was prepared as described above, except the procedure was not performed aseptically and no analgesic was administered. **Figure 5** displays the experimental configuration

and a schematic including the flow parameters.

An initial incision was made at the right caudal region of the spinotrapezius muscle and the incision was extended along the distal portion of the muscle to reveal the muscle. The connective tissue above the spinotrapezius muscle was removed using micro-dissection scissors and fine forceps. During the procedure, the muscle was continually irrigated with physiological salt solution (PSS) at a flow rate of ~2 mL min^{-1}. The PSS was deoxygenated with 5% CO_2 – 95% N_2 and warmed to 35° C.

After the connective tissue was removed, the intravital microscope (Microscan MIcrovision Medical) was placed over the muscle to visualize the microvasculature of the muscle, and the surgical stage was adjusted to locate the area of interest on the muscle. A 30-minute equilibration period was undergone between dissection and the addition of the first dilator as well as in between the addition of each dilator to allow the vessels to return to rest. In the pilot study, different concentrations, from 10^{-8} to 10^{-4}, were added with a micropipette to the PSS for both Bradykinin and Isoproterenol, with a five minute wait period between each concentration—recordings were taken at each time point—and the reactivity of the terminal arterioles was determined. In the second study, the optimal amount, 10^{-5} M, of each vasoactive agent (bradykinin, isoproterenol, and sodium nitroprusside) was added to the PSS to study the reactivity of the arterialized collateral capillaries. Ten-second videos were recorded before, and five minutes after the addition of each reagent. The same procedure was followed for the sham side, following the lateral feed artery to examine terminal arterials of similar size. Upon completion, the mouse was euthanized by cervical dislocation.

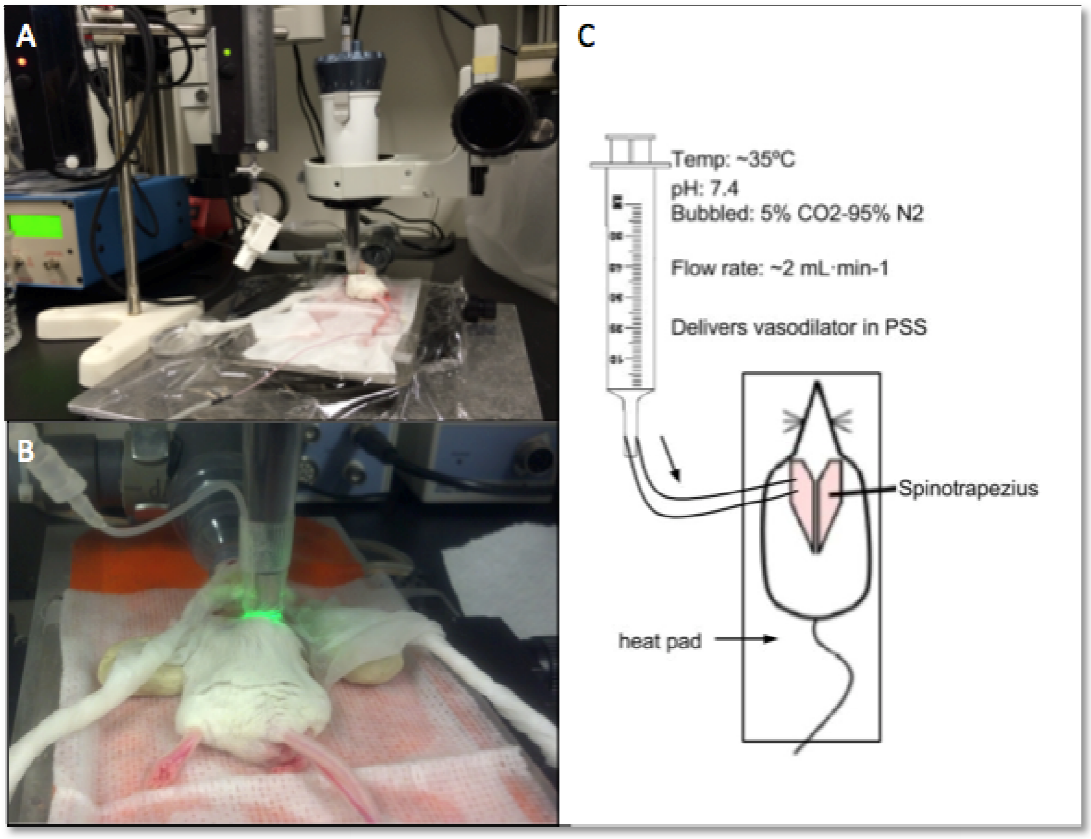

Figure 5: Intravital microscopy superfusion set-up. A. Representative image of preparation with microscan and heated syringe. B. Close up of mouse with heat pad and Microscan. C. Schematic of flow.

Imaging and Statistical Analysis

Automated Vascular Analysis (AVA) software was used to stabilize the videos and conduct measurements of the arteriole diameter. Measurements were performed on the stabilized images to determine the difference in arteriole diameter before and after exposure to the reagents. Statistical analysis was performed on the data generated from the images in the AVA software to compare the diameters for the varying concentrations of reagents and time points as well as to compare the diameter changes on the operated and sham sides. One-way ANOVA tests were performed on the data, with post-hoc Tukey's tests used to determine the location of significant differences.

RESULTS

The first objective of the project was to complete a pilot study that involved measuring the vasodilation of small terminal arterioles in response to increasing doses of isoproterenol (SMC dep) and bradykinin (EC dep), 10^{-7} to 10^{-4} M, and sodium nitroprusside (SMC dep) to identify the lowest concentration necessary to achieve a maximum dilation, **Figure 6**. Swiss-Webster mice from the Cal Poly colony were used to conduct the pilot study due to similarity to Balb/c mice and easy availability.

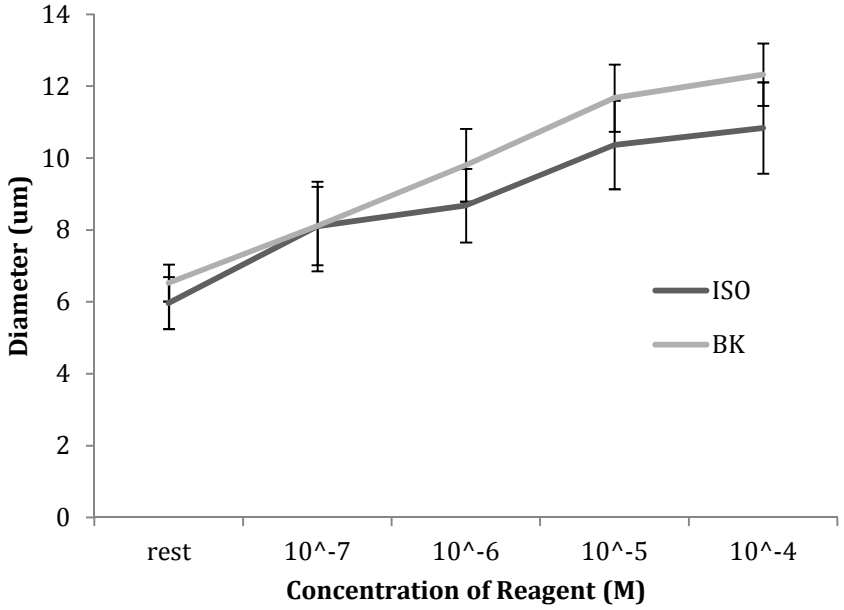

Figure 6: Superfusion dosage response in unoperated mice. Line graph displays change in diameter (µm) before and after log-increasing doses of bradykinin (BK) and isoproterenol (ISO) (n=5).

Based on the results from the pilot study, we determined that the optimal concentration for both bradykinin and isoproterenol was 10^{-5}M, which represented the plateau of the dose response "S" curve, in which diameter did not significantly increase with the next log dilution (determined after running repeated measures ANOVA for each reagent). Sodium nitroprusside

was previously determined to have optimal vascular reactivity at 10^{-5} M [9]. After completing the pilot study, the main objective of this project was to test the hypothesis that smooth muscle-dependent vasodilation is impaired in immature arterialized capillaries. At day 7 following lateral feed artery ligation, we measured vasodilation of arterialized collateral capillaries in response to 10^{-5} M BK, ISO and SNP, **Figure 7**.

On the sham side, the vasoactive agents induced significant vasodilation from rest (7 ± 0.5 μm) when exposed to BK (12 ± 0.5 μm), ISO (13 ± 0.5 μm) and SNP (12 ± 0.5 μm). The arterialized collateral capillaries on the ligated side also experienced vasodilation from rest (7 ± 0.5 μm) when exposed to BK, ISO, and SNP. Although the ACCs vasodilated in response to the vasoactive agents, for ISO there was less dilation on the ligated side (10 ± 1 μm) compared to dilated diameter on the sham side (13 ± 0.5 μm). For SNP, there was also a significant decrease in dilated diameter on the ligated side (10 ± 0.5 μm) compared to the sham side (12 ± 1 μm), **Figure 7**.

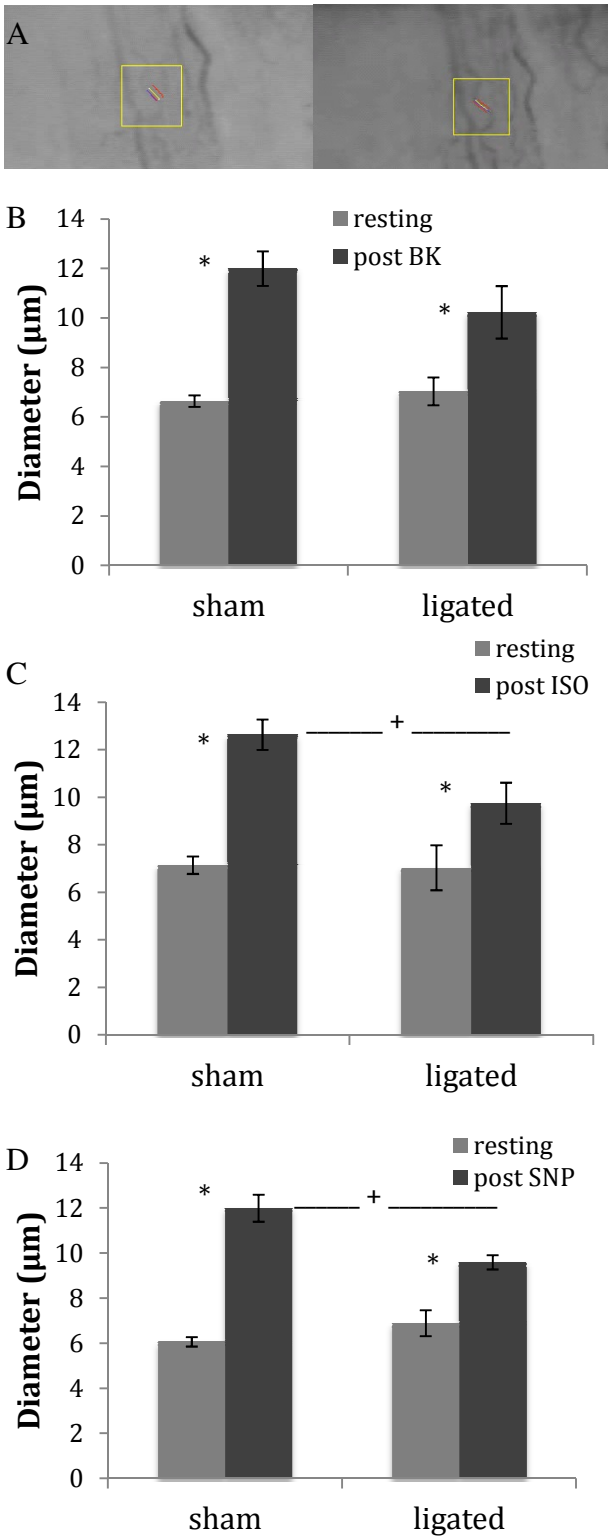

Figure 7: Vascular reactivity of arterialized collateral capillaries. A. Representative image of an arterialized collateral capillary pre and post exposure to BK. B. Diameters (um) pre and post exposure to 10^{-5} M BK (n=7). C. Diameters (µm) pre and post exposure to 10^{-5} M ISO (n=7). D. Diameters (µm) pre and post exposure to 10^{-5} SNP (n=5). * $p<0.05$ difference from baseline. + $p<0.05$ difference between sham and ligated

The percent change of each vessel diameter was compared on the sham and ligated side to determine if there was impaired vasodilation in the immature arterialized collateral capillaries, **Figure 8**.

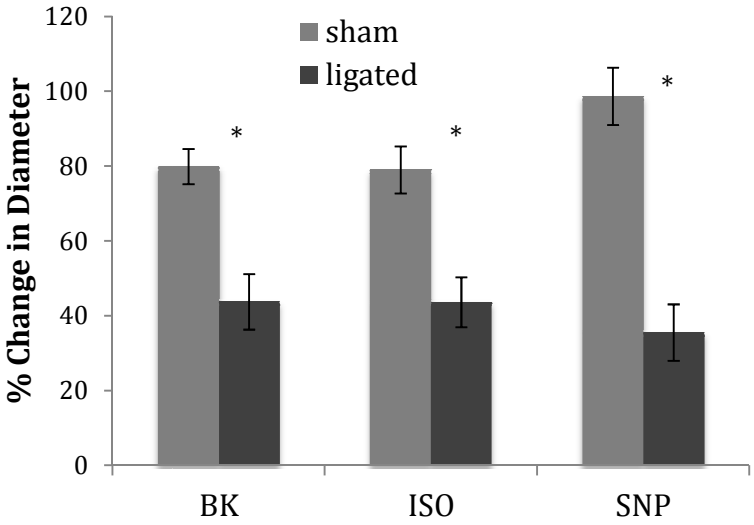

Figure 8: Relative reactivity of arterialized collateral capillaries. Percent change in diameter before and after exposure to BK (n=7), ISO (n=7), and SNP (n=5). * $p<0.05$ for sham versus ligated.

When exposed to bradykinin, the terminal arterioles on the sham side had a larger percent change in diameter (80 ± 5) compared to the arterialized collateral capillaries on the ligated side (44 ± 7). In response to the isoproterenol, the terminal arterioles on the sham side had a larger percent change in diameter (79 ± 6) compared to the ACCs on the ligated side (44 ± 7). Sodium nitroprusside also elicited a larger percent change in diameter of the terminal arterioles (99 ± 7) compared to the ACCs (36 ±8).

DISCUSSION

Clinical Significance

Peripheral arterial occlusive disease can lead to chronic ischemia and intermittent claudication due to a plaque buildup in limb arteries. Collateral vessels can act as natural bypasses to provide improved blood to this ischemic region flow for patients with POAD, but pre-existing collateral networks are not always present in patients with ischemia. In animal models of chronic ischemia, arterialization of collateral capillaries, by arteriogenesis, can restore blood flow to the ischemic region. However, to provide sufficient blood flow, the collateral vessels must exhibit functional vasodilation, the vessels must be able to dilate in response to increased tissue metabolism. Arterialized collateral capillaries in Balb/c mice have impaired vasodilation 7 days post artery ligation, but dilate normally at day 21 [9]. The goal of this study was to confirm the impaired smooth muscle cell-dependent responses in the arterialized collateral capillaries.

Vascular Reactivity

Endothelial cell-dependent vasodilation was tested using bradykinin, an endothelial-dependent agonist. The immature arterialized collateral capillaries exposed to bradykinin showed significant, but impaired vasodilation compared to the terminal arterioles on the sham side. Bradykinin targets the GPCRs on the endothelial cells lining the arteriole wall and through the GPCR cascade, causes the formation of nitric oxide, which is released into the smooth muscle cells and causes relaxation. It is unknown which part of the pathway is inhibited and further testing of the endothelial-dependent pathway should be tested. Specifically the GPCR cascade in the endothelial cell, separate from the smooth muscle cell response, should be tested

to confirm impairment of the endothelial-dependent vasodilation. The results do not confirm or refute impaired vascular reactivity due to endothelial cell impairment.

Smooth muscle cell-dependent agonists, isoptorterenol and sodium nitroprusside, which cause vasodilation in microvasculature, were applied onto immature arterialized collateral capillaries at day 7 following an artery occlusion. The decrease in vascular reactivity in the ACCs compared to the terminal arterials indicates that smooth muscle cells are impaired in immature arterialized collateral capillaries 7-days post ligation. SNP and ISO cause vasodilation through different pathways in the smooth muscle cells; SNP breaks down into NO which increases cGMP and leads to the phosphorylation of MLCK, whereas ISO targets the beta-adrenergic receptor to activate adenylyl cyclase, increase cAMP and ultimately phosphorylate the MLCK to prevent contraction and induce vasodilation. The results indicate that both types of pathways that lead to vasodilation in the immature arterialized collateral capillaries are impaired. SNP had a larger trending change in percent difference from sham to ligated, but showed no significant difference compared to ISO, which could indicate that the pathways have various levels of impairment.

By testing the vascular reactivity of the arterialized collateral capillaries in response to bradykinin, isoproterenol, and sodium nitroprusside, we were able to determine that the smooth muscle cells at day 7 following an artery ligation contribute to impaired vasodilation, but it is unknown what causes impairment of vascular smooth muscle cells.

Smooth Muscle Cell Phenotype

Vascular smooth muscle cells (VSMC) are categorized based on contractile properties, with considerable phenotypic diversity in various vessels throughout the body [5]. VSMC phenotype is related to varying developmental origins and can be influenced by the outward

remodeling of the vessel due to arteriogenesis [18, 16]. Structural remodeling could contribute to different smooth muscle contractile phenotypes and reduced vasodilation capacity.

Arterialization of the capillaries occurs when capillaries recruit smooth muscle cells and gain the ability to dilate and contract, but the contractile ability is not fully functional at the onset of arteriogenesis. Changes in the myosin light chain and myosin heavy chain during the remodeling phase could enhance the vessels response to external stimuli with respect to the regulation of blood flow [5]. Different vessels express various phenotypes that allow for different levels of vasomotion and vascular smooth muscle phenotype may be driven by innervation, load, or other varying external signal [5].

Future Work

To understand why smooth muscle cells are impaired in ACCS at day 7 following an artery ligation, future research should study the remodeling of the vascular smooth muscle cells and the changes in the myosin light chain and myosin heavy chain isoforms during arteriogenesis. Impairment of the vasodilation could be due to the change in phenotype and migration of SMCs to the capillaries during arterialization and remodeling [18].

Examining vascular remodeling at the cellular level would help explain the lack of functional vasodilation by understanding how the cells are arranged and changing between day 7 and day 21 after arterialization of the collateral capillary.

Limitations

One limitation in this experiment was the accuracy of the dosage response curves created in the first part of the study. There was a large standard error associated with the vascular reactivity of both the BK and ISO reagents on terminal arterioles in the unoperated mice. It is possible that in the arterialized collateral capillaries there was normal vasodilation at

lower doses and only impaired vasodilation at higher doses, thus changing the interpretation of the data in the first part of the study. An inaccuracy in selecting the optimized dosage could have resulted in a discrepancy in the percent change of the ACC diameter.

Summary

Functional vasodilation in immature arterialized collateral capillaries is impaired, to some extent, due to smooth muscle cell impairment at day 7 following occlusion of the lateral feed artery. The impairment of smooth muscle cells in the newly formed collateral vessels can hinder the recovery of ischemia and fail to match blood flow with tissue metabolic demand. If the smooth muscle cell recruitment and regeneration is better understood, it may be possible to induce the formation of mature, functional arterialized collateral capillaries after an occlusion. Therefore, understanding the cause of impairment is important to more effectively increase blood flow to the ischemic region, reduce the duration of impaired vasodilation, and may provide the foundation for future treatments for patients with PAOD.

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APPENDIX

Vascular Reactivity checklist and protocol

Date_____ **Intravital Microscopy with Superfusion** Initials _____

Mouse Information

DOB: _____
Sex: _____
Tag: _____
Genotype/strain: _____
Cage: _____
Weight: _____

Materials

___1. forceps (1)
____2. fine forceps (1)
____3. ultrafine forceps (2)
____4. fine scissors (1)
____5. ultrafine scissors (1)
____6. 60mL syringe
____7. bubbler (2)
____8. volumetric flask
____9. Kim wipes
____10. Vasodilator/vasoconstrictor
____11. 20X PSS
____12. 20X $NaHCO_3$
____13. non-sterile cotton swabs
____14. non-sterile cotton gauze
____15. 10mL Graduated cylinder
____16. stopwatch

Instrument Preparation

____17. Turn on ultrasonic bath to 45ºC
____18. Transfer 50mL of 20x PSS into 1L volumetric flask
____19. Transfer 50mL of 20x $NaHCO_3$ into 1L volumetric flask
____20. Dilute PSS & $NaHCO_3$ to 1L with 18MΩ H_20
____21. Place 1L volumetric flask in water bath, using weight if necessary
____22. Fill syringe in syringe heater with 50mL of 1x PSS & turn on syringe heater
____23. Place thermister in syringe heater
____24. Weigh out or thaw vasodilator

Surgery preparation

____25. Weigh animal in weight boat
____26. Place animal in anesthesia box
____27. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 $l \cdot min^{-1}$
____28. Anesthetize animal w/ 5% isoflurane
____29. Reduce flow rate to 0.5-1.0 $l \cdot min^{-1}$ and the isoflurane to 1-3%
____30. Lay animal supine on preparation bench with nose in nose-cone
____31. Use trimming clippers & depilatory cream to remove hair superficial to spinotrapezius
____32. Transfer mouse to stage on FST heat pad
____33. Insert rectal probe and set thermo-controller to 35°C
____34. Open stopcock on delivery tubing to check flow rate of superfusion solution and use thermister to measure temperature
____35. Adjust flow rate or temperature to achieve ~2mL·min^{-1} and ~35ºC at tip

Measurement preparation

____36. Make an incision (1cm) at the caudal end of the spinotrapezius
____37. Extend the incision cranially to the fat pad, creating a horse shoe incision
____38. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
____39. Maximize visibility of muscular branch- minimize contact/trauma of the artery

Intravital Microscopy

____40. Recheck flow rate and temperature of supefusion solution
____41. Place microscan in stand and insure proper connectivity to computer.
____42. Open AVA and create new folder containing date of procedure and make current folder.
____43. Select capture on AVA main menu for microscan imaging
____44. Label patient I.D. with the number of patient first followed by left or right hind limb or sham for control hind limb.

EX: 25LeftACh8

____45. Locate muscular branch and adjust microscan for best resolution.
____46. Position superfusion delivery tubing with ball-bearing manipulator at microscan lens to ensure flow over the artery
____47. Place kim-wipe wick on side of animal (avoid contact with muscle)
____48. Use AVA to measure muscular branch diameter after 30 minutes of stabilization.
____49. Ensure that 60mL syringe contains 60mL of PSS
____50. Add dose of first vasodilator agent
____51. Allow superfusion to flow for 5 minutes and record video in the final minute
____52. Empty 60mL syringe, rinse with 18MΩ H_2O
____53. Refill syringe with 60mL of PSS and repeat superfusion with 2nd and 3rd vasoactive agents
____54. Repeat procedure on the contralateral limb

Analysis

____55. Open analysis section in AVA and open file of interest
____56. Set Frames from 0 to 160 and stabilize file
____57. Analyze vessel diameter by manually drawing diameter and chaining sections together
____58. Record results in provided table

Post-Experiment

____59. Cervical dislocation to euthanize animal

Date_____ **Intravital Microscopy with Superfusion** Initials _____

____60. Rinse superfusion line (bubbler, superfusion tubing, syringe, & side-arm flask) with water (flush tubing w/ syringe)

____61. Flush superfusion line with 1M HCl & rinse with water

____62. Flush superfusion line with 18MΩ H_2O

Limb 1

Left or Right

BK Resting Diameter_____

BK volume & concentration_____

BK dose 1 Diameter_____

ISO Resting Diameter_____

ISO volume & concentration _____

ISO Diameter_____ ____

SNP Resting Diameter_____

SNP volume & concentration _____

SNP Diameter _____ ___

Limb 2

Left or Right

BK Resting Diameter_____

BK volume & concentration_____

BK dose 1 Diameter_____

ISO Resting Diameter_____

ISO volume & concentration _____

ISO Diameter_____ ____

SNP Resting Diameter_____

SNP volume & concentration _____

SNP Diameter _____ ___

Notes

_____ _____
_____ _____
_____ _____
_____ _____
_____ _____
_____ _____
_____ _____