VASCULAR REACTIVITY IN IMMATURE ARTERIALIZED CAPILLARIES

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ABSTRACT

VASCULAR REACTIVITY IN IMMATURE ARTERIALIZED CAPILLARIES

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Peripheral arterial occlusive disease (PAOD) is a globally prevalent cardiovascular disease in which atherosclerotic plaques narrow arterial lumen diameters and restrict blood flow to down stream tissues. The impact of these occlusions can be mitigated by collateral vessels that connect parallel arterial branches and act as natural bypasses to maintain perfusion. Some patients with PAOD may not have robust collateral networks to accommodate ischemic tissues in the event of an occlusion and, therefore, may be more susceptible to hypoxia and tissue necrosis. In animal models that lack collateral arterioles, capillaries can arterialize and form functional collaterals; however, in the early stages of development, they do not exhibit functional vasodilation in response to muscle contraction. We explored the mechanism of impaired functional vasodilation in arterialized capillaries by testing endothelial-dependent vasodilation (acetylcholine), endothelial-independent vasodilation (sodium nitroprusside), and endothelial-independent vasoconstriction (norepinephrine). First, we performed pilot studies to generate dose-response curves to each of these agents in spinotrapezius arterioles. The optimal concentration that produced the most robust vascular responses was 10^{-5} M for all three agents. In the following study, the spinotrapezius feed artery was ligated in Balb/C mice, a strain that lacks native collaterals, to stimulate the development of arterialized capillaries and determine their vascular reactivity. Although vasodilation and vasoconstriction in arterialized capillaries seven days post-surgery were impaired compared to terminal arterioles of similar size in unoperated animals, the vessel diameter changes were still significant. The comparable impairment in both endothelial-dependent and endothelial-independent vasodilation indicates that vascular smooth muscle cells are still developing, rearranging, or both, and are not yet fully capable of regulating diameter in immature arterialized capillaries. Identifying factors that improve the functionality of smooth muscle cells in the arterialized capillaries may be applied to improve patient prognosis during ischemic events in vasculature lacking pre-existing collaterals.

Keywords: arteriogenesis, arterialization, ischemia, peripheral arterial occlusive disease,

vasodilation, spinotrapezius

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"When we try to pick out anything by itself, we find it hitched to everything else in the universe."
– John Muir

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I. INTRODUCTION

Clinical Relevance

Chronic ischemia—a restriction of bloody supply to tissue—impairs arterial function in the microcirculation [2]. Specifically, chronic ischemia due to peripheral arterial occlusive disease (PAOD) impairs vasodilation in the limb skeletal muscles (**Figure 1**) [11]. In 2012, approximately 8 million Americans had PAOD, a number that

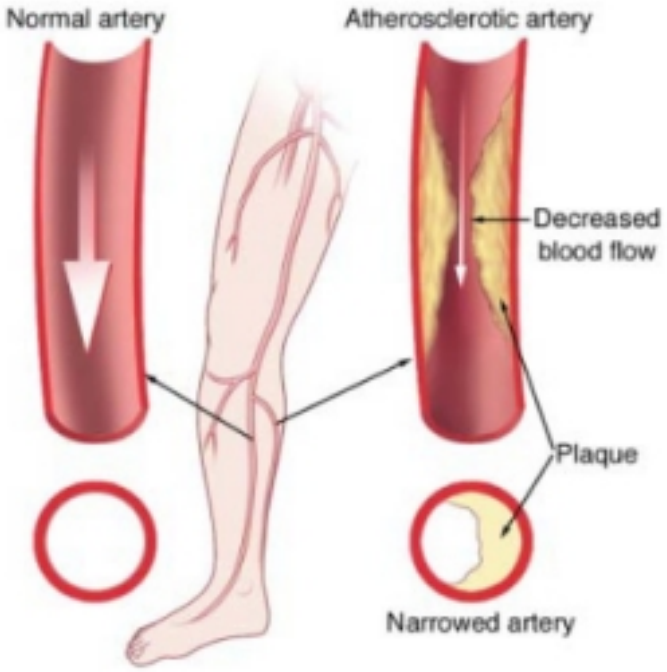

Figure 1. Atherosclerosis in PAOD, where narrowed and hardened arteries reduce blood flow to limbs [3].

has been increasing for the past decade. The most common symptom of PAOD is intermittent claudication, or hypoxic pain during locomotion, which is amplified during exercise [3]. The ischemic tissues do not receive sufficient oxygen or nutrients, and a chemical imbalance leads to cramping and discomfort [3]. In PAOD patients, this discomfort is extreme and prevents any further immediate exercise.

Once PAOD has been diagnosed, initial treatments include lifestyle modifications such as cessation of smoking, integration of regular physical activity, and adjustments in diet. If lifestyle modifications are ineffective, pharmacotherapy with anti-thrombogenic medications such as aspirin can be administered to prevent thrombus formation on the atherosclerotic plaque(s). In severe cases, bypass surgery and percutaneous interventions, including angioplasty, can be used to restore blood flow downstream of the atherosclerotic plaque as a last resort [3,7]. Unfortunately, not all patients are candidates for surgery, percutaneous interventions may fail due to restenosis, and current pharmacotherapy medications often have undesirable side effects. Therefore, developing alternative methods to restore blood flow to ischemic zones is necessary to improve PAOD-patient prognosis and treatment.

Previous Work

Stimulating the development of collateral networks is one potential alternative for restoring blood flow in patients that are not candidates for bypass or percutaneous interventions. Collateral networks improve patient prognosis by redirecting blood flow in the event of arterial occlusion. With a collateral, or a vessel connecting two parallel arterial segments in series, nutrient-rich blood has an alternative path to reach downstream tissues that would, otherwise, become anoxic [26]. When blood flow is redirected and shear stresses increase due to the occlusion, collateral vessels enlarge via arteriogenesis, in which vessels outwardly remodel and incorporate a thicker layer of smooth muscle cells [9]. Historically, arteriogenesis was only thought to be possible in pre-existing collateral arterioles, and not all animal strains have robust collateral networks to support arteriogenesis by this historical definition. The variation in collateral

density between strains of mice can be extrapolated to humans such that some patients may be genetically more susceptible to ischemia.

Two strains of mice that exemplify differences in collateral networks are C57BL6 and Balb/C. C57 is a commonly used strain of mouse in research [25] and has a high density of collaterals, while the Balb/C strain has a lower or nonexistent density of collaterals, depending on the tissue. In Balb/C mice, however, upstream arterial occlusion induces outward remodeling on a smaller scale, involving the arterialization of so-called "collateral capillaries" that anatomically connect two vascular trees. Although they typically have high resistances that prevent their function as true collaterals, these capillaries can recruit smooth muscle cells and develop into arterioles [9,30]. The newly developed arterialized capillaries act as collaterals to reperfuse ischemic tissues; however, these new vessels are functionally impaired in their early stages [6].

Specifically, functional vasodilation is absent seven days after occlusion of upstream arteries in the spinotrapezius muscle of murine models [6]. Interestingly, at 21 days post-ligation, these arterialized capillaries regained functionality and demonstrated normal vasodilation following muscle contraction, even trending towards a greater than normal response. Regaining vasodilatory capabilities is important because, when it is impaired, these arterialized capillaries fail to meet the demands of downstream tissue when metabolism increases, for instance, during locomotion or exercise [4].

Because the impairment is temporary, the initial dysfunction may be attributed to immaturity of the vascular smooth muscle cells within the outward remodeling phase [20]. The cause of early impairment in vessel function is unknown: whether it relates to smooth muscle cell function, endothelial cell function, or both. Understanding

impairment in microcirculation, specifically in new collateral vessels resulting from occlusions of PAOD, could provide knowledge to support the development of more efficacious therapies to mediate and resolve one of the most prevalent health issues in the world today.

Purpose of Current Study

The purpose of this study was to determine the mechanism of impaired vasodilation in immature arterialized capillaries. Specifically, we tested the hypothesis that impaired functional vasodilation can be explained by vascular smooth muscle cell (VSMC) dysfunction. To test this hypothesis, arterialized capillaries were exposed to acetylcholine (ACh), sodium nitroprusside (SNP), and norepinephrine (NE) via superfusion of a physiological salt solution seven days post-ligation. These agents allowed us to evaluate endothelial-dependent vasodilation via acetylcholine and smooth muscle-dependent vasodilation via sodium nitroprusside. Endothelial-independent vasoconstriction via norepinephrine was also evaluated to confirm that the VSMCs were contractile.

Acetylcholine (ACh) is a neurotransmitter that leads to vasodilation by diffusing from synaptic clefts into endothelial cells and activating a G-protein coupled receptor (GPCR) cascade that stimulates endothelial nitric oxide synthase (eNOS) to then produce NO [14]. NO binds to smooth muscle cell guanylyl cyclase receptors, which increases cyclic guanosine monophosphate (cGMP) production, thereby activating protein kinase G (PKG). PKG closes calcium channels, opens potassium channels, and phosphorylates myosin light chain kinase, decreasing intracellular calcium levels, inducing hyperpolarization, and inhibiting contraction, respectively. This reduces cross-bridge

cycling and relaxes the muscle to ultimately achieve vasodilation [14]. Sodium nitroprusside (SNP) triggers vasodilation by breaking down into nitric oxide (NO) and other components, relaxing the smooth muscle directly (**Figure 2**).

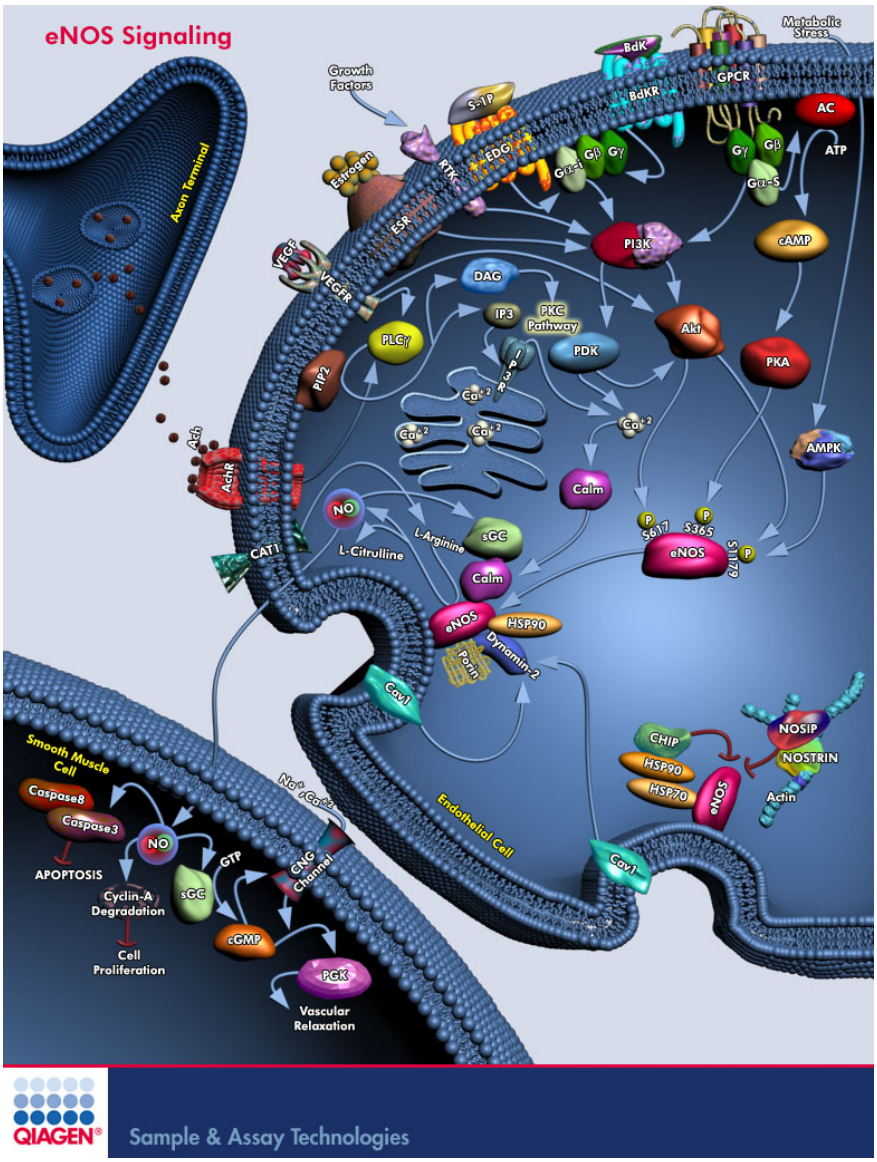

Figure 2. Cell signaling for smooth muscle relaxation [24]. Regardless of the initial stimulus, vascular smooth muscle ultimately vasodilates in response to NO.

Because SNP bypasses the endothelial activation of NO, SNP is considered a smooth muscle-dependent vasodilator, and ACh is considered an endothelial-dependent

vasodilator. Norepinephrine is a neurotransmitter that activates alpha-1 adrenergic receptors on the smooth muscle cells to increase intracellular calcium, increase cross-bridge cycling, and constrict the vessel (Figure 3) [14].

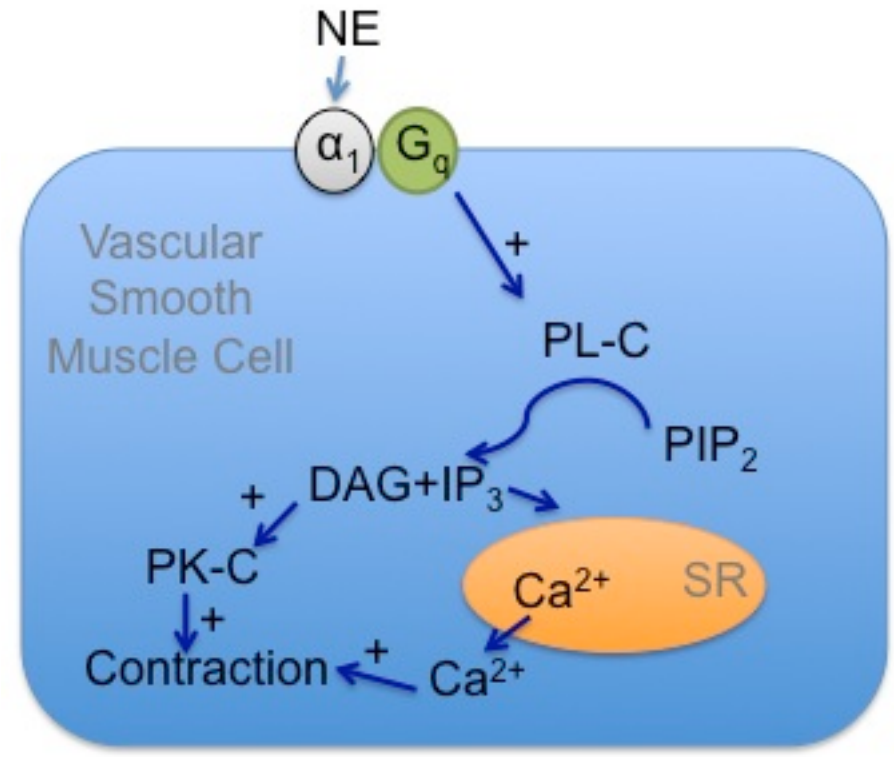

Figure 3. Cell signaling for smooth muscle contraction. Increasing intracellular calcium within the smooth muscle allows for increased cross-bridge cycling.

The adrenergic receptors activate phospholipase C to break phosphatidylinositol 4,5-bisphosphate (PIP2) down into inositol trisphosphate (IP3) and diacylglycerol (DAG). DAG and IP3 increase intracellular calcium directly and by activating phosphokinase C, respectively.

We exposed murine spinotrapezius muscles to each of the vasoactive agents to determine which cell type impairs vasodilation in the arterialized capillaries. Equal responses from both the terminal arterioles in the sham side and arterialized capillaries in

the ligated side would demonstrate a lack of dysfunction or damage in the corresponding vessel; however, failure to respond or difficulty responding implies that either one or both cell types are impaired. We hypothesize that the smooth muscle is immature so that we expect equal relative impairment in response to ACh and SNP on the ligated side. Once the contributors to vascular impairment are specified, we may be able to identify factors to quicken and support the development and maturation of arterialized capillaries. Less time with dysfunctional collaterals will reduce tissue susceptibility to ischemia, as perfusion will more effectively meet tissue metabolic demand. As found in different strains of mice, there may be a distinct variation in human vasculature that affects our aptitude to respond to ischemia. Individuals with robust collateral networks are better equipped to redirect blood flow around an occlusion and avoid strokes and myocardial infarctions [30]. By facilitating efficient development of these collateral networks, we may improve prognoses of patients with less advantageous vasculature to cope with diseases like PAOD.

Specific Aims

Specific Aim 1: To assess the reactivity of terminal arterioles in unoperated mice with a normal density of pre-existing collaterals to determine optimum reagent concentrations and to anticipate expected responses.

Specific Aim 2: To test the hypothesis that smooth muscle-dependent vasodilation is impaired in arterialized capillaries at day-7 following spinotrapezius feed artery ligation.

II. METHODS

Animal Care and Housing

Male swiss webster and Balb/C mice were housed in microisolator cages within temperature controlled rooms in the University Vivarium for the duration of the experiment where 12 hour light and dark cycles were maintained. All mice were provided with food, water, bedding, a plastic "mouse house," and a plastic tube. Balb/C mice had no more than three other cage-mates. These mice were cared for and utilized under the guidelines specified by the Cal Poly State University SLO Institutional Animal Care and Use Committee.

Vascular Reactivity with Intravital Microscopy – Dose Response

All equipment that contacted the solutions was rinsed at the end of every procedure. This equipment included two bubblers, two 60 mL syringes, a flow meter, small sections of 1/8" tubing, luer connections, and tips. Physiological salt solution (PSS) was prepared the day of each procedure from stock solution with 131.9 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 2 mM $CaCa_2$, and 18 mM $NaHCO_3$ mixed in a 1 L volumetric flask with 1 L of ohmic water. The flask was heated and deoxygenated in a water bath set to 50ºC with 5% CO_2-95% N_2 bubbled into it. pH was kept at ~7.4 and checked with a pH meter. The solution was manually transferred to heated 60 mL syringes as needed to maintain an effluent flow rate of ~2 $mL \cdot min^{-1}$ and temperature of ~35ºC. ACh, SNP, and NE were diluted from 10^{-3} Molar stock solutions to 10^{-5} Molar.

Each mouse was initially anesthetized using 5% isoflurane in oxygen in an induction chamber. The mice were, then, transferred to a preparation bench with isoflurane reduced to ~1-3% and maintained at ~3 $l \cdot min^{-1}$ via nose cone throughout the preparations and procedure. The hair on the anterior dorsal aspect of each mouse was

removed with clippers and depilatory cream. Following skin preparations, each mouse was transferred to a heat pad in the prone position.

Internal temperatures of each anesthetized mouse were kept at 35ºC via heat pad and rectal thermistor. Following an initial skin incision at the caudal end of the spinotrapezius, the skin was retracted and connective tissue removed to expose the muscle. The exposed tissue was continually irrigated with PSS from the heated syringes, and any area not irrigated by the PSS was covered with plastic wrap to prevent desiccation and to minimize atmospheric oxygen exchange.

Terminal arterioles in the spinotrapezius were identified on the intravital microscope and video was captured of the baseline diameters of the terminal arterioles after a 30 minute stabilization period. Vasodilator and vasoconstrictor agents were administered via micropipettes and a graduated cylinder to the area through the syringe and tubing at 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} Molar concentrations, in that order. After each vasoactive agent was added, a 5-minute waiting period was utilized to allow the vessels to equilibrate before recording the videos in the final minute of each respective waiting period. At the end of the experiment, the mouse was euthanized using cervical dislocation.

Spinotrapezius Lateral Feed Artery Ligation

To stimulate the arterialization of capillaries, the lateral spinotrapezius feed artery was ligated. Mice were anesthetized and hair was removed as described above. Buprenorphine analgesic (0.075 $mg \cdot kg^{-1}$) was subcutaneously administered prior to the surgery. To prevent corneal desiccation, veterinary ophthalmic ointment was applied to the eyes of the mice. A skin incision was made above the cranial, lateral edge of the

spinotrapezius where it meets with the fat pad. Phosphate buffered saline (PBS) was frequently applied to prevent desiccation. The fat pad cranial and superficial to the spinotrapezius muscle was blunt dissected to expose the lateral edge. Between the fat pad that lies deep to the spinotrapezius and the lateral edge of the muscle; the spinotrapezius artery/vein pair was identified and the artery was isolated and ligated with free strands of 6-0 silk suture. The skin incision was closed with 7-0 polypropylene suture and analgesic was subcutaneously administered immediately after the surgery to minimize discomfort as the animal recovered. For two days following the ligation, the mice received oral buprenorphine (0.01 mg/mL) mixed in with the water.

Vascular Reactivity with Intravital Microscopy

7 days following each ligation surgery the spinotrapezius muscle was re-exposed. The same superfusion protocol was followed, as described above, but each vasodilator agent was only administered to the area once at 10^{-5} concentrations. Terminal arterioles were analyzed on the sham side and arterialized capillaries were analyzed on the ligated side.

Imaging and Statistical Analysis

The images/videos were analyzed using AVA software to compare diameters at the respective time points and concentrations via percent differences, standard deviation, and standard error. Significance between diameters and percent changes before and after reactivity and between the sham and ligated sides was rated against a 0.05 p-value using paired and homoscedastic statistical t-tests.

III. RESULTS

Average dosage response curves were calculated for each of the three vasoactive agents in swiss webster strain mice to determine the concentration necessary to elicit a maximal response: a maximum vasodilation in response to acetylcholine (ACh) and sodium nitroprusside (SNP), and a maximum vasoconstriction in response to norepinephrine (NE) (**Figure 3**).

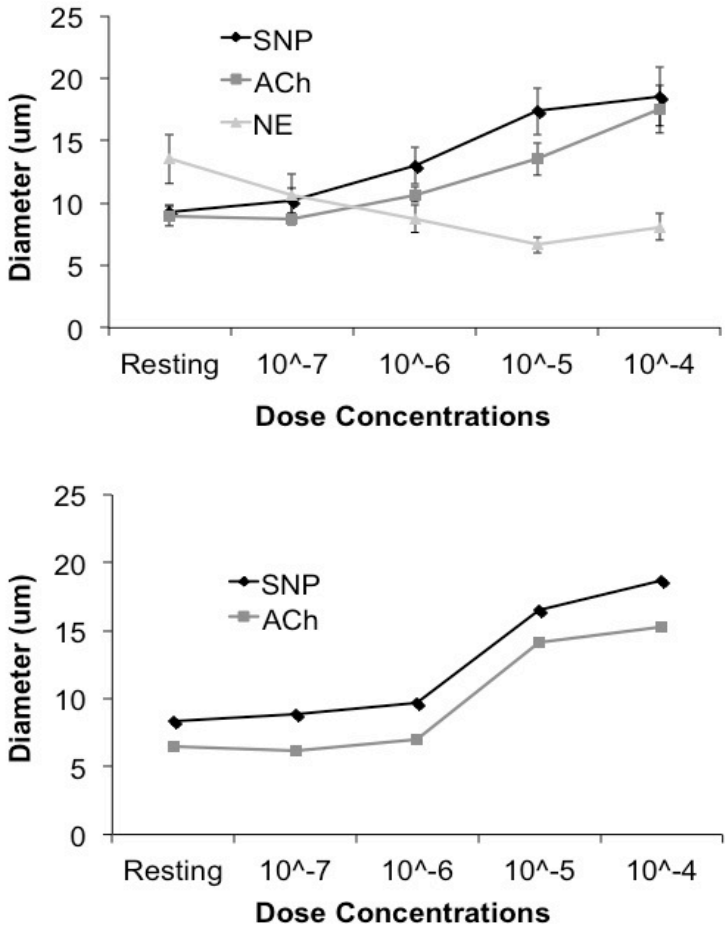

Figure 4. Superfusion dosage responses in terminal arterioles of unoperated animals. A) Diameter in microns of Swiss-Webster strain pre and post exposure to 10-fold dilutions of ACh (n=7), SNP (n=9), and NE (n=5). B) Diameter in microns of Balb/C pre and post exposure to 10-fold dilutions of ACh and SNP (n=1).

The diameter responses from both the Swiss-Webster strain (high density of collaterals) and the Balb/C strain (low density of collaterals) followed a sigmoid curve pattern. The

resting diameters and responses at each concentration are comparable between the strains. Vessels reached a maximal vasodilation or vasoconstriction at 10^{-5} molar concentration in response to each vasoactive agent. Only one dosage response was performed for the Balb/C strain so that statistics could not be evaluated between the Swiss-Webster and Balb/C diameters.

We utilized 10^{-5} molar concentrations of each agent to elicit maximal vasodilation and vasoconstriction responses. To compare reactivity between arterialized capillaries and terminal arterioles of approximately the same size in unoperated muscles, vascular reactivity was assessed in the spinotrapezius muscles of mice with ligated and unligated (sham) feed arteries seven days post-surgery. The muscles were exposed to acetylcholine and sodium nitroprusside to observe vasodilation responses, and to norepinephrine to observe vasoconstriction responses. Vessel diameters were measured in terminal arterioles before and after ACh and SNP application; however, larger arterioles were measured following norepinephrine application in attempts to measure accurate diameters during vasoconstriction. Nearly all vessels on the unoperated sides constricted to a point at which luminal diameter was absent.

ACh (endothelial-dependent) and SNP (endothelial-independent) both significantly increased vessel diameters in the terminal arterioles of the sham side and arterialized capillaries in the ligated side to roughly the same degree; however, the terminal arterioles dilated significantly more than did the arterialized capillaries (**Figure 5A,B**). NE (endothelial-independent) significantly decreased vessel diameters in the sham and ligated sides; however, the terminal arterioles constricted significantly more than did the arterialized capillaries (**Figure 5C**).

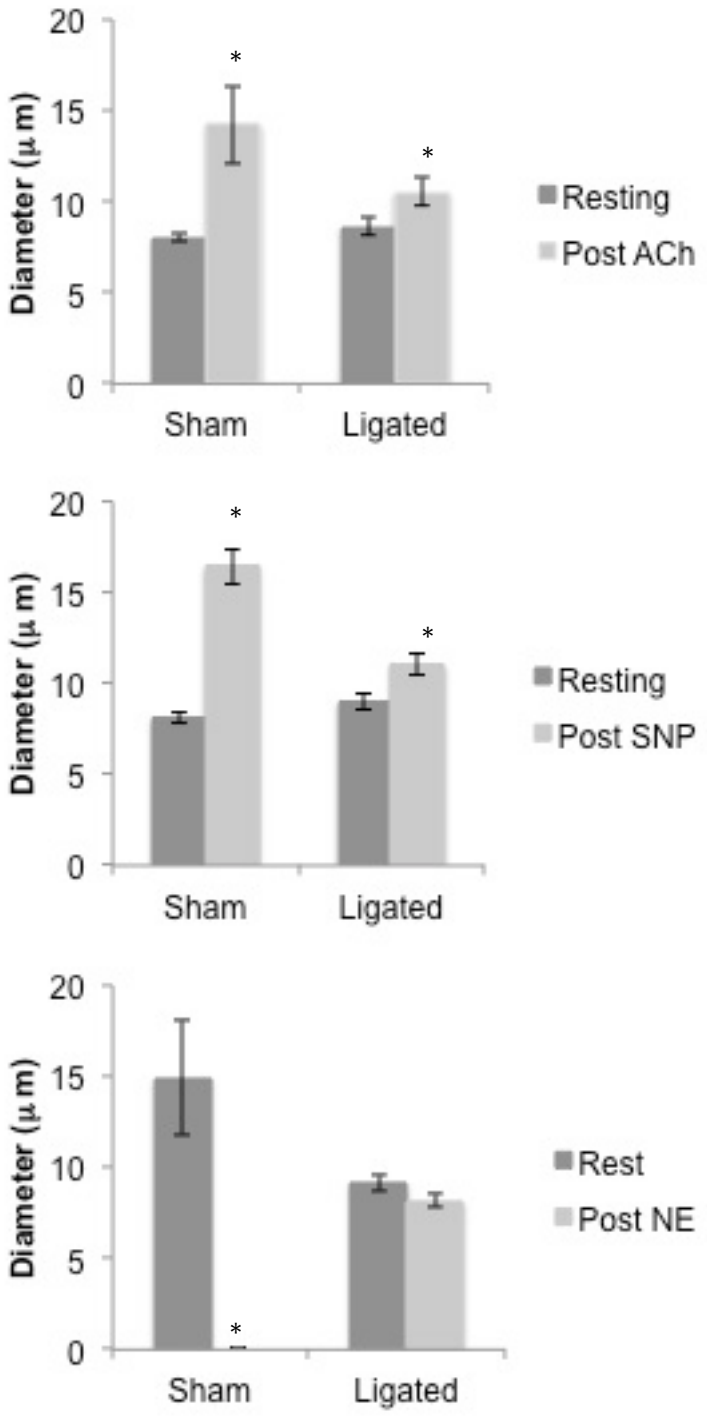

Figure 5. Superfusion Day 7 - Terminal arterioles on sham and arterialized capillaries on ligated side. A) Diameters in microns pre and post exposure to 10^{-5} ACh (n=7). **B)** Diameters In microns pre and post exposure to 10^{-5} SNP (n=7). **C)** Diameters In microns pre and post exposure to 10^{-5} NE (n=6); * indicates p <= .05 using a paired students t-test

For the control arterioles, ACh induced a significant vasodilation from rest 8.0±0.8 μm vs. dilated 14.2±6.4 μm, SNP induced a significant vasodilation from rest 8.1±0.9 μm vs. dilated 16.4±2.9 μm, and NE induced a significant vasoconstriction from rest 14.9±9.4 μm vs. dilated 0.0±0.0 μm. For the arterialized capillaries, ACh induced a significant vasodilation from rest 8.6±1.0 μm vs. dilated 10.5±1.7 μm, SNP induced a significant vasodilation from rest 9.0±1.1 μm vs. dilated 11.0±1.6 μm, and NE induced a vasoconstrictive trend from rest 9.2±1.2 μm to 8.2±1.0 μm.

When comparing the percent changes in diameter between the sham and ligated sides after exposure to vasoactive agents, there were significant differences for each vasoactive agent. The two vasodilators, ACh and SNP, both increased the percent change diameter in the sham side significantly more than in the ligated side. The percent change induced by ACh on the sham vs. ligated side was 72.7±23.2% vs. 23.0±4.9%. The percent change induced by SNP on the sham vs. ligated side was 104.0±10.8% vs. 23.2±5.5%. The vasoconstrictor, NE, significantly decreased the percent change in diameter in the sham as compared to the ligated side from -100.0±0.0% vs. -10.7±7.8% (**Figure 6**).

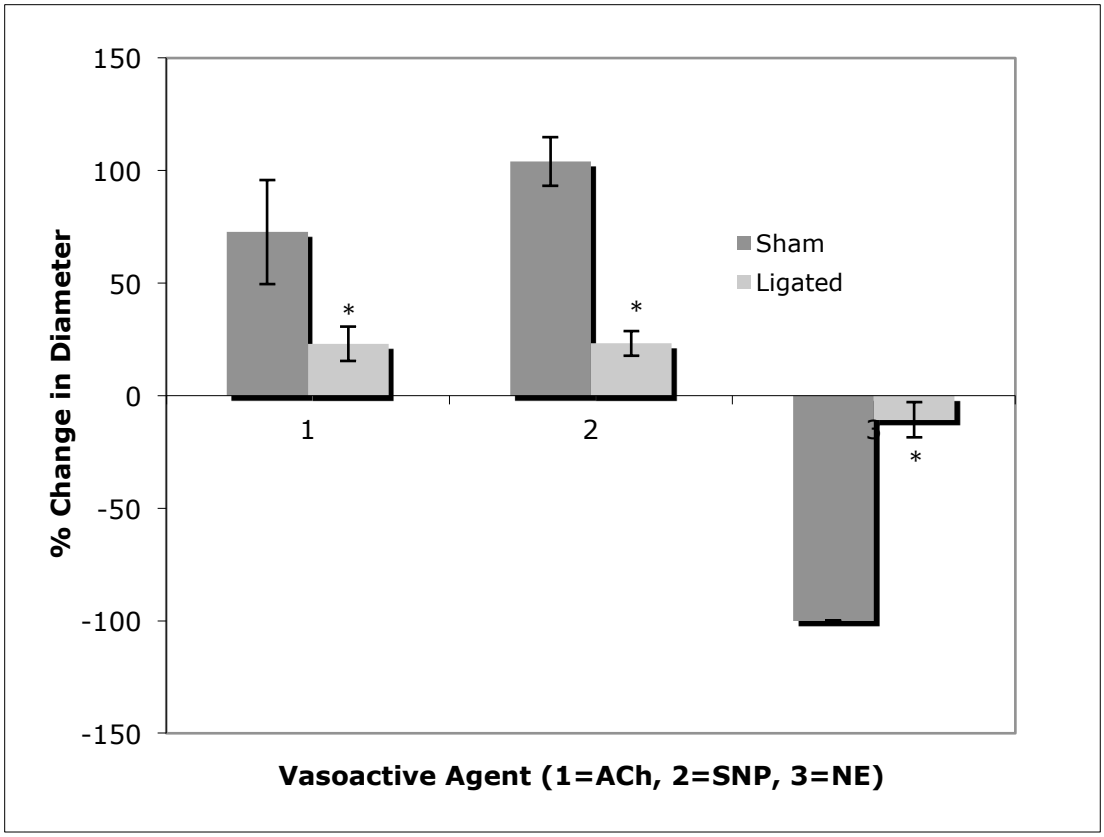

Figure 6. Relative vasoreactivity of the arterioles in the spinotrapezius. Percent changes between the sham and ligated sides in their respective responses to 10^{-5} M ACh and SNP in the terminal arterioles, and 10^{-5} M NE in the arterioles. P =< 0.05 for all vasoactive agents; n=4.

SNP has a tendency to induce a greater vasodilation than ACh, though not significantly. Although the vasoactive agents cause significant changes in diameter to the arterialized capillaries, these changes are significantly less than those in terminal arterioles.

IV. DISCUSSION

Collateral vessels can maintain perfusion in downstream tissues that would, otherwise, be ischemic in the event of a conduit artery occlusion. These collaterals respond to increased shear stress by outwardly remodeling and increase blood flow as a result of that process; however, collaterals can only outwardly remodel if they exist in the vasculature. The arterialization of collateral capillaries in animals lacking pre-existing collateral arterioles can compensate enough to re-establish blood flow to an ischemic vascular tree [6,9]; however, to match blood flow with tissue demand in the reperfused areas, collaterals and/or arterialized capillaries need to be able to sufficiently vasodilate. Although the arterialized capillaries acquire smooth muscle cells within seven days following the spinotrapezius lateral feed artery ligation, functional vasodilation is absent at this time point [6,7,9]. In this study, we investigated possible causes of this impairment by observing endothelial cell-dependent and smooth muscle cell-dependent responses of arterialized capillaries seven days following the ligation of the spinotrapezius feed artery in mice lacking collaterals. We established a dosage response curve to identify the optimal concentrations to induce vasodilation and vasoconstriction.

We also found that, although the arterialized capillaries did not respond to functional vasodilation at seven days post-ligation, they did significantly dilate or constrict to respective vasoactive agents. This discrepancy can be explained by the fact that these arterialized capillaries may not yet be a part of the conduction circuit within the vasculature. In normally functioning arterioles, local signals to dilate or constrict are conducted across the endothelial cells to change the diameter of upstream arterioles to effectively increase flow to the initial local stimulus [13]. Capillaries may not naturally be included in this circuit, or may not have the appropriate directionality to continue a

signal within the circuit, so it may take longer than seven days for them to fully arterialize and adapt to it. The vasoactive reagents were a more direct application to the endothelial and smooth muscle cells within the arterialized capillaries than the electrode stimulation of the muscles to instigate distinct diameter changes before the arterialized capillaries develop into part of the conduction circuit.

Both endothelial cells and smooth muscle cells affect the remodeling process of arterioles. On a smaller scale, we hope to reveal more specifically how they contribute to the impaired vascular reactivity of the arterialized capillaries. At 7 days post-ligation, the arterialized capillaries demonstrated significantly less reactivity as compared to the terminal arterials of the non-ligated "sham" side. The arterialized capillaries dilated less in response to the vasodilators and constricted less in response to the vasoconstrictors. Because endothelial-independent vasodilation mechanisms were impaired to the same degree as endothelial-dependent mechanisms, we can associate at least part of the dysfunction with smooth muscle cells; however, we do not have sufficient evidence to associate causation with endothelial cell impairment. Potential causes of impairment include smooth muscle cell phenotype, mechanoadaptation, ECM remodeling, and lack of innervation. Mechanoadaptation and ECM remodeling are thought to eventually increase the reactivity of smooth muscles, at 28 days for example, but may initially inhibit proper smooth muscle function [6]. The decreased vasoconstriction may also be explained by a lack of innervation of the arterialized capillaries.

Endothelial Cell Impairment

Endothelial cell impairment may contribute to the impaired vasodilation and vasoconstriction in the arterialized capillaries; however, testing endothelial impairment

would require measuring the production of relaxing factors of the endothelial cells. Because this was not included in the study, we cannot explain the impairment by dysfunction of the endothelial cells.

Smooth Muscle Cell Phenotype

Mechanoadaptation entails the reorientation of vascular smooth muscle cells to accommodate a change in flow within a blood vessel. These mechanical changes are instigated by changes in shear stress, for example, perhaps, when blood supply is redirected as a result of an occlusion. In cases where pre-existing collateral vessels exist, blood flow will follow the path of least resistance and travel through the existing network, around the occlusion (**Figure 7**). The pre-existing collaterals experience increased shear stress following the ligation and consequent vasoactive autacoids are released. These autacoids include growth factors and vasodilators, like endothelium-derived hyperpolarizing factor and prostaglandins, all of which contribute to the function and structure of vascular smooth muscle cells. [15] The smooth muscle cells have acute and chronic responses. During acute responses, smooth muscle cells utilize actin and myosin cross-bridge cycling to perform vasoconstriction; however, calcium sensitization and actin filament remodeling are also acute processes that can inhibit vasoconstriction responses. In a longer-term, chronic context, smooth muscle cells have been observed to physically rearrange. [15]

Smooth muscle cells must be present for mechanoadaptation to occur. Because capillaries do not naturally have smooth muscle cells, they must develop and/or recruit the cells for mechanoadaptation to be a potential means of remodeling.

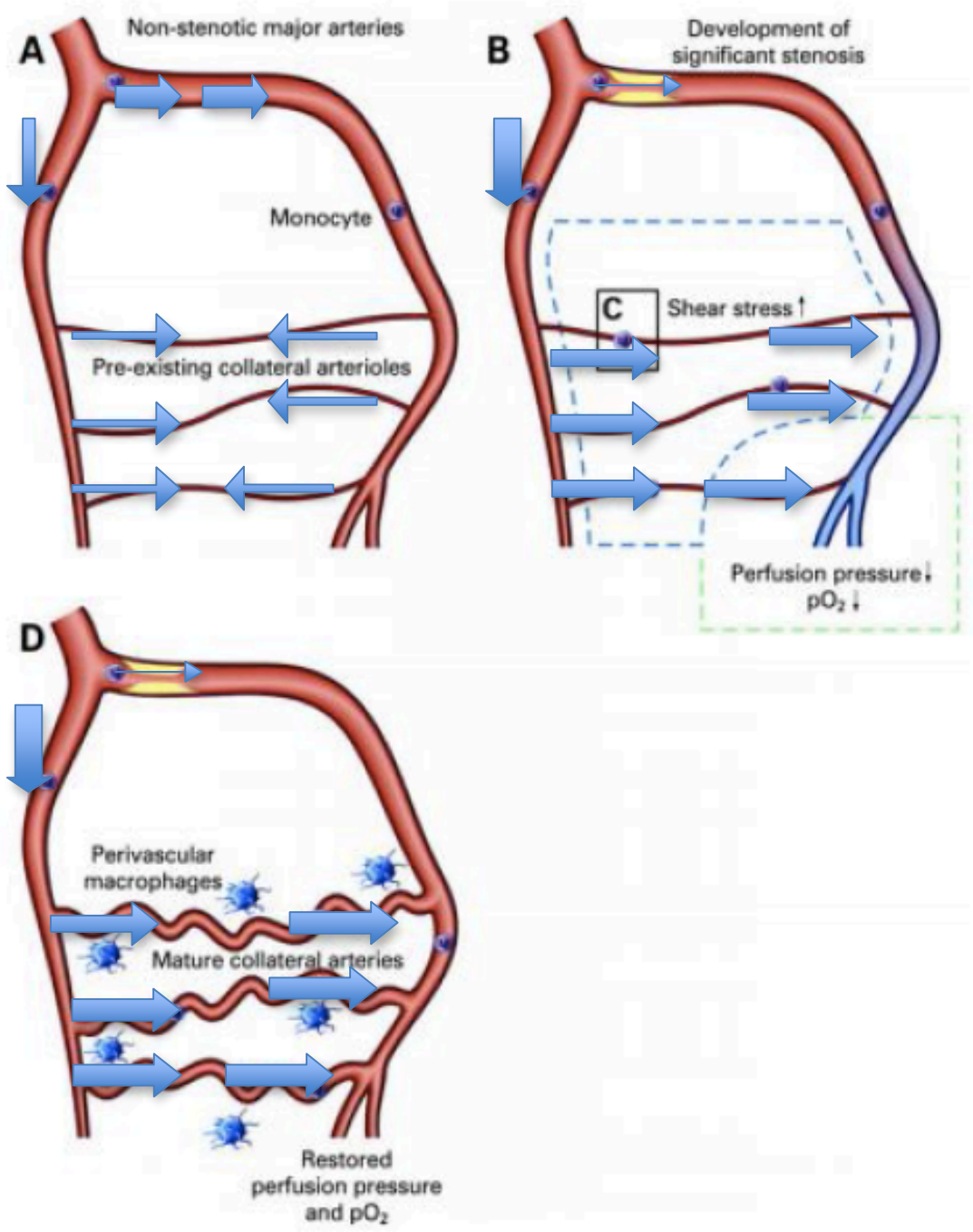

Figure 7. Blood flow and shear stress shift inducing collateral vessel development. [10]

Vascular development revolves around the communication between endothelial and smooth muscle cells in regulating vascular formation, stabilization, remodeling, and function via factors such as hepatocyte growth factor (HGF) and angiopoietin-1 (Ang1)

[17]. Ang1, which regulates genes in ECs, is involved in recruiting the SMCs and maturing the vasculature, so that the success of the vascular development depends on paracrine communication with ECs. Vascular development also depends on endocrine factor processing [17].

It is possible that the development and recruitment of the smooth muscle cells is contributing to the impairment because the cells are growing and/or adjusting. Periovascular cells can be recruited from the nearby arterioles or from the immediately surrounding tissue [22,28]. In any case, the cells will take time to arrange and, potentially, to further develop into fully functional cells. For example, if the fibroblasts are developing into SMCs, they may not yet have sufficient levels of actin and myosin to properly contract or dilate. Thus, the immaturity of the arterialized capillaries would impair vascular reactivity.

Future work to expand on this research could entail ex-vivo examination of the arterialized capillaries to determine their precise cellular constituents. Observing vascular wall components of capillaries before, during, and after the remodeling process would provide a more clear depiction of how the cells are arranging and changing. Once we better understand the process, we may be able to stimulate or control it in vivo.

Extracellular Matrix Remodeling

In addition to the rearrangement of smooth muscle cells, the extracellular matrix-integrin-cytoskeletal axis is activated in response to long-term exposure to shear stress. In this axis, cell-cell and cell-extracellular matrix connections are strengthened to maintain vascular wall structural integrity; it is also dynamically involved in controlling vasoreactivity. [15] The responses in the ECM revolve around strengthening and

stiffening the vascular wall, a remodeling method thought to make constriction and/or dilation more efficient. [15]

Outward remodeling has previously been observed in preexisting collaterals and vessels that already have the smooth muscle cells and ECM interactions to begin with. Smooth muscle cells do not naturally exist in capillaries, as vasoconstriction and vasodilation are not functions of that vessel type. When the spinotrapezius feed artery is ligated in a muscle containing few or no collaterals, blood flow redirects through collateral capillaries that connect arterial branches from other nearby feed vessels to accommodate the lack of perfusion. These collateral capillaries enlarge, and may remodel in a similar fashion to the preexisting collaterals. The results from this study point to early stages of smooth muscle cell maturation as a cause for the impaired vasoreactivity. The smooth muscle cells are likely immature in development and positioning so that they are not as receptive and/or responsive to vasoactive agents.

The ability of capillaries to arterialize provides many possibilities for individuals lacking pre-existing collaterals to reperfuse ischemic tissues in the event of an arterial occlusion. These developed capillaries provide new bypass routes for blood to travel around the occlusion and reach areas downstream from the blockage. Further understanding of the mechanisms of this development may expose specific elements that are involved; these elements could be exogenously introduced to elicit or quicken the development process. The sooner blood returns to an ischemic area, the less time the tissues lack oxygen, nutrients, and the potential for necrosis.

Lack of Innervation

Capillaries do not naturally have smooth muscle cells or innervation from the sympathetic nervous system, as dilation and constriction to regulate blood flow are not capillary functions. Though it can also cause dilation through β-adrenergic receptors, SNS stimulation typically causes vasoconstriction in skeletal muscle arterioles by activation of α-adrenergic receptors [27]. While norepinephrine (NE) is a very common constricting factor, cells may not be sensitive to it if they are not regularly exposed to the neurotransmitter. The insensitivity to NE could be due to decreased expression of the α-adrenergic receptor, a complete lack of innervation, insufficiently organized actin and myosin preventing effective contractions.

Limitations

One limitation to this study is that dosage response curves were only performed on one replicate of the Balb/C strain. It is possible that the reactivity of the Balb/C strain varies enough from that of the Swiss-Webster strain that the concentrations used were not maximal, on average. The latter instance would have affected the data by resulting in smaller diameters during vasodilation and larger diameters during vasoconstriction, effectively reducing the percent change in diameter after exposure to each of the vasoactive agents. In future studies, dosage response curves could be calculated with more replicates of the Balb/C strain to confirm the optimal concentrations for NE, SNP, and ACh.

Another limitation of this study is that other, natural stimuli are not necessarily accounted for. Sometimes, the vessels are dilated by other factors, such as muscle twitching, so that the baseline measurements may not reflect true resting values. When the muscles twitch, the local vessels dilate and vessels in other branches constrict,

varying the baseline to exaggerate either a difference or a similarity. The effects of muscle twitching are mitigated by continual irrigation with PSS and covering exposed tissue with plastic wrap when not in use. However, these measures do not prevent random muscle twitching and may not eliminate other stimuli. A more developed protocol for obtaining and monitoring baseline values may allow for more accurate measurements. For example, more effectively preventing muscle twitching with a different saline solution or cover, or minimizing the presence of other stimuli with longer equilibration periods may improve baseline accuracy.

Summary

Vascular reactivity in arterialized capillaries is, at least in part, impaired by smooth muscle cells, which are presumably immature. This immaturity may lead to dysfunction because the smooth muscle cells are not yet fully-aligned, may not yet contain normal levels of actin and myosin, or may express lower levels of vasoactive receptors. By understanding the mechanisms behind the impairment, we may be able to minimize the duration and extent of the impairment to more quickly and effectively increase perfusion to downstream tissues. An extrapolated application of this could potentially be to induce or quicken the development of new collateral vessels. Manipulating the arterialization of capillaries may improve PAOD patient prognosis by more quickly and appropriately providing and improving blood flow to ischemic tissues downstream of an occlusion.

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