VASCULAR REACTIVITY IN NEWLY-FORMED AND MATURE ARTERIALIZED COLLATERAL CAPILLARIES

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ABSTRACT

Vascular Reactivity in Newly-Formed and Mature Arterialized Collateral Capillaries

Sara Hellstrom

Peripheral arterial occlusive disease (PAOD) is a globally-prevalent cardiovascular disease in which atherosclerotic plaques narrow arterial lumen diameters and restrict blood flow to downstream 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. In animal models that lack collateral arterioles, capillaries that connect terminal arteriolar segments can arterialize and form functional collaterals following an ischemic event; however, in the early stages of development, vasodilation is impaired. We explored the mechanism of impaired vasodilation in arterialized collateral capillaries (ACCs) and pre-existing collaterals (PECs) by evaluating endothelial-dependent vasodilation and endothelial-independent reactivity at day seven following the ischemic event. We also evaluated functional vasodilation in mature ACCs and PECs at day 21 by applying vasodilation inhibitors during the electrical stimulation of muscle contraction. Arterial occlusion was performed by ligating the cranial-lateral spinotrapezius feed artery in Balb/C mice, a strain that either lacks native arteriolar collaterals or contains a single collateral arteriole (~50% of mice), as opposed to the C57Bl/6 strain, which each contain 10 or more collateral arterioles. At seven days post-surgery, both vasodilation and vasoconstriction were impaired in ACCs when compared to terminal arterioles of similar size in unoperated limbs, but still exhibited significant changes when compared to baseline. The comparable reactivity in both endothelial-dependent and independent vasodilation at day-seven in ACCs indicates that vascular smooth muscle cells are likely responsible for the impairment, as they may still be developing, rearranging, or both, and are not yet fully capable of regulating diameter in immature ACCs. However, by 21 days post-ligation, ACCs regained the capacity to dilate in response to muscle contraction, and utilized similar vasodilation pathways as control vessels. At seven days post-ligation, PECs had impaired endothelial-independent dilation, but successful endothelial-dependent dilation, indicating the use of alternative pathways to dilate. Unlike ACCs, the PECs never completely restored vasodilation capabilities by day 21, which may be due to a variation in smooth muscle phenotype, sensitivity to vasoactive agents, and/or limited growth factor expression. For future work, evaluating collateral formation and vasodilation in a diseased model and investigating molecular variations in the smooth muscle may yield additional knowledge that can improve therapies for patients during ischemic events.

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

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“Life isn’t about waiting for the storm to pass, it’s about learning to dance in the rain (that is your thesis).”
– Nancy Sathre-Vogel

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Clinical Relevance

Ischemia—an insufficient blood supply to tissue—impairs arterial function in the microcirculation (9). Specifically, chronic ischemia due to peripheral arterial occlusive disease (PAOD) impairs vasodilation in the limb skeletal muscles (**Figure 1**) (4, 9). In 2012, approximately 8 million Americans had PAOD, a number that has been

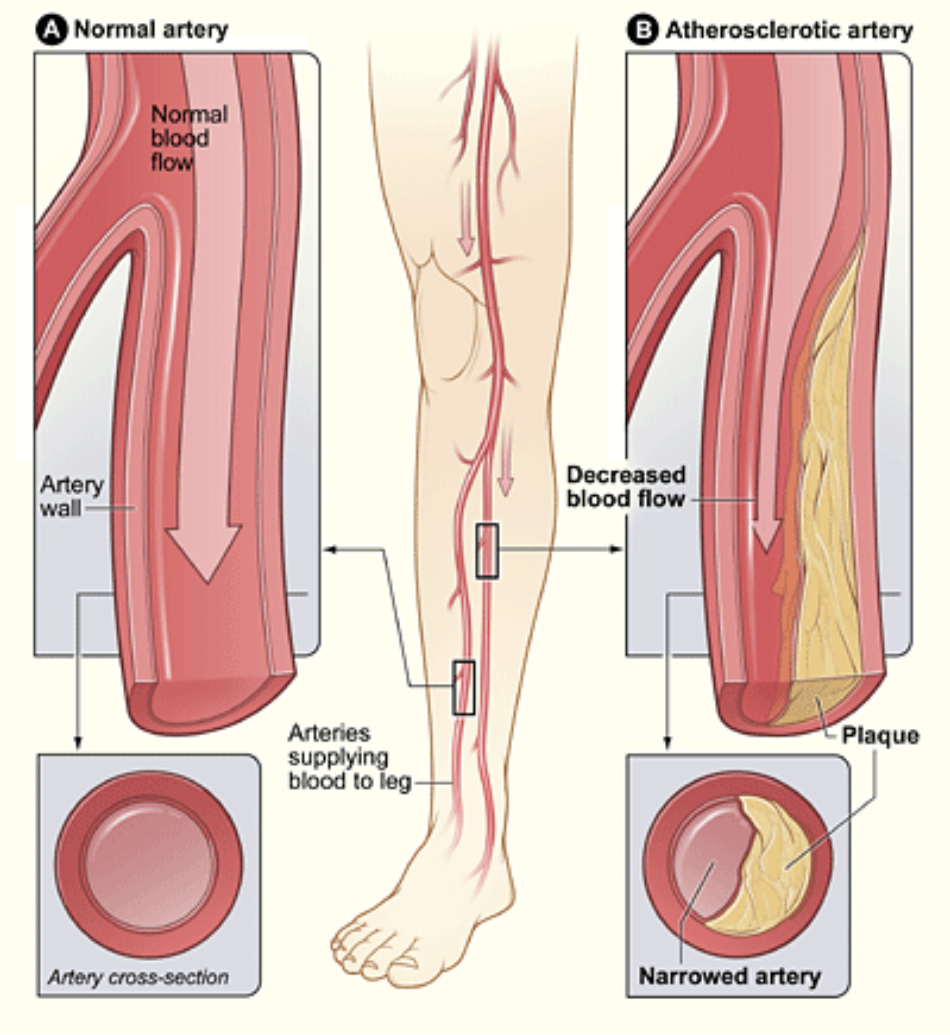

Figure 1. Atherosclerosis in PAOD. In PAOD, narrowed and hardened arteries reduce blood flow to limbs (47).

increasing for the past decade (10). PAOD has three general causes: inflammation, atherosclerosis, and thrombus formation. Inflammation is a physiological response to

damaged tissue or cells in which cytokines and growth factors are released, and leukocytes are recruited to the area. When the influx of cells and heightened activity continue, the process transitions to chronic inflammation and can cause damage to the nearby tissue and leads to stenosis, or narrowing of the artery (29, 38). Atherosclerosis develops when the lipids, inflammatory cells, and extracellular matrix components accumulate under the endothelium of arteries (55). Low-density lipoproteins (LDLs) are oxidized and phagocytized by macrophages, which accumulate and form foam cells. Foam cells and smooth muscle cells migrate into the sub-endothelial space and can obtrusively shape the lumen, leading to stenosis (**Figure 2**) (55). Thrombus formation is often associated with atherosclerosis, as the foam cells collect and become a risk for embolism when the deposits develop the potential to burst into the lumen (38). Additionally, the endothelium becomes dysfunctional in atherosclerotic conditions and fails to maintain vascular homeostasis by releasing factors to regulate reactivity, smooth muscle proliferation and migration, fibrinolysis, and thrombogenesis, further contributing to the risk of thromboembolism (15).

The most common symptom of PAOD is intermittent claudication, or hypoxic pain during locomotion, which is amplified during exercise (10, 64). Cramping and discomfort develop in the ischemic tissues from insufficient oxygen and nutrient exchange, resulting in metabolite accumulation (10, 54). In PAOD patients, this discomfort is extreme and prevents any further immediate exercise.

Patients are diagnosed with PAOD by symptom assessment and measuring an ankle-brachial index (ABI) or vascular imaging, such as ultrasound, magnetic resonance angiography (MRA), and/or computed tomographic (CT) scan. ABI measures a

comparative blood pressure between ankle and arm while ultrasound, MRA, and CT are imaging tests that visualize plaque accumulation (10). Once PAOD has been diagnosed, initial treatments include lifestyle modifications such as cessation of smoking, integration of regular physical activity, and adjustments in diet.

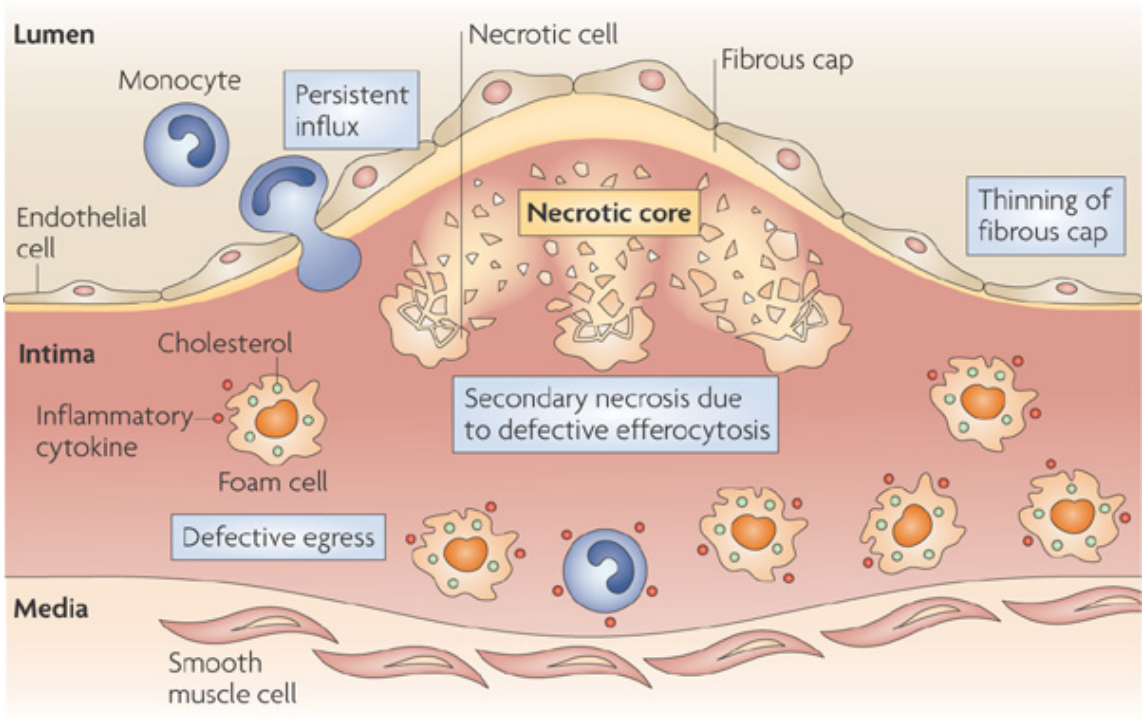

Figure 2. Neointimal formation during atherogenesis (72)**.** Leukocytes infiltrate the intimal space and necrotic cells form fibrous cap to develop atherosclerotic plaque.

If lifestyle modifications are ineffective, pharmacotherapy with anti-platelet medications such as aspirin can be administered to prevent thrombus formation on the atherosclerotic plaque(s). Antiplatelet medications reduce the risk of serious vascular events in patients with PAOD by 23% (21). Other medicines, like angiotensin-converting enzyme (ACE) inhibitors, slow atherogenesis and improve peripheral blood pressure to the extent that mortality, myocardial infarction, and stroke rates are reduced (21). In severe cases of PAOD, bypass surgery and percutaneous interventions, including

angioplasty, are used to restore blood flow downstream of the atherosclerotic plaque (10, 21). Bypass is a relatively invasive surgery in which a section of a blood vessel is transferred from an artificial source or from another area of the body to provide an alternative route for blood to flow around the occlusion. Angioplasty, a less invasive surgery than bypass, pushes the occluded vessel radially outward via intraluminal catheter, restoring blood flow in the downstream vascular network. Unfortunately, not all patients are candidates for surgery, percutaneous interventions may fail due to restenosis, and current pharmacotherapy medications often have undesirable side effects such as diarrhea, neutropenia, and thrombotic thryombocytopenic purpura (21). 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 a potential alternative for restoring blood flow in patients that are not candidates for bypass or percutaneous interventions. Collateral networks are natural bypasses that 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 (62). 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 (25). Historically, however, 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 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 when a low number of collateral networks are present.

Two strains of mice that exemplify differences in collateral networks are C57Bl/6 and Balb/C. C57 is a commonly used strain of mouse in research (60) 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, upstream arterial occlusion induces outward remodeling – specifically, arterialization – of so-called "collateral capillaries" that anastomose terminal arterioles of two adjacent vascular trees. Although their high resistances prevent their initial function as true bypass collaterals, these capillaries recruit smooth muscle cells and outwardly remodel into arterioles (25, 58). The newly developed arterialized capillaries have a lower resistance to blood flow and can act as bypass collaterals to reperfuse ischemic tissues. Unfortunately, these new vessels are functionally impaired in their early stages, failing to dilate and increase flow in response to muscle contraction (12, 13). Regaining vasodilatory capabilities is important because, when they are impaired, these arterialized capillaries fail to meet the demands of downstream tissue when metabolism increases, for instance, during locomotion and exercise. Specifically, functional vasodilation is absent seven days after occlusion of an upstream feed artery in the spinotrapezius muscle of mice (13). Functional vasodilation is restored, however, 21 days following occlusion (13).

Because the impairment is temporary, the initial dysfunction may be attributed to immaturity of the vascular smooth muscle cells within the outward remodeling phase (42). The cause of early impairment in vessel wall function is unknown: whether it relates

to smooth muscle cell function, endothelial cell function, or both. Understanding the function of new collateral vessels may allow us to support their development and reduce the duration and scale of their impairment. Although these new vessels are seemingly mature at 21 days, three weeks may be too long for patients to recover effectively from an ischemic event, as in PAOD or coronary heart disease (CHD) (80). Thus, accelerating collateral development could mediate and resolve one of the most prevalent health issues in the world today; however, we first need to understand the mechanisms of vasodilation dysfunction to progress with such research.

Specific Aims and Hypotheses

The overall goal of this project is to determine the mechanisms of vasodilation and impaired vasodilation during the development of arterialized capillaries and remodeling of pre-existing collaterals. Understanding these mechanisms will provide a foundation for developing improved therapeutic agents for patients suffering from Peripheral Arterial Occlusive Disease (PAOD) with a dendritic, ischemia-prone vasculature, as seen in the Balb/C mouse strain.

Specific Aim 1: To test the hypothesis that smooth muscle-dependent vascular reactivity is impaired in arterialized collateral capillaries and pre-existing collaterals at day-7 following spinotrapezius feed artery ligation.

Specific Aim 2: To test the hypothesis that functional vasodilation in mature arterialized collateral capillaries and pre-existing collaterals at day-21 following feed artery ligation utilize similar pathways as control vessels.

Chapter II. REACTIVITY AT DAY-7

INTRODUCTION

Collaterals are beneficial in the event of arterial occlusion by providing patient circulation with an alternative path to reach downstream tissue (62, 79). The spinotrapezius muscle within the Balb/C mouse strain serves as a model for an extreme case of low-density collateral networks within the vasculature (11, 12, 79). In this model, the capillaries connecting dendritic arterial branches arterialize when an upstream artery is occluded to support a circulatory bypass route (25). Seven days following spinotrapezius feed artery ligation, the arterialized collateral capillaries that have formed do not vasodilate in response to muscle contraction (13). This inability to dilate is likely due to the arrangement and immaturity of smooth muscle cells (26, 42). Smooth muscle is not typically present around capillaries, so it is possible that the cells require more than seven days to fully develop and orient themselves as the cells are either recruited from upstream arterioles or differentiate from existing perivascular cells (25, 48, 68).

The purpose of this study was to test the hypothesis that smooth muscle-dependent vascular reactivity is impaired in arterialized collateral capillaries (ACCs) at day-7, which required the optimization of reagent concentrations to elicit maximal responses for endothelial-dependent (acetylcholine), and endothelial-independent (sodium nitroprusside, norepinephrine, papaverine, sodium hydrogen sulfide) vasoactive agents. Additionally, pre-existing collaterals (PECs) were analyzed in the same manner as the ACCs to compare reactivity of collateral vessels that have a more established smooth muscle layer. We anticipated a generalized smooth muscle-based impairment in both vessel types, as the smooth muscle layer is adapting in both scenarios to accommodate the ischemic event by developing an alternative route for blood flow.

Smooth-muscle dependent vascular reactivity was determined by comparing the effects of endothelial-dependent and endothelial-independent vasodilators and an endothelial-independent constrictor. Equal or lesser responses with endothelial-independent reagents, as compared to endothelial-dependent reagents, would imply smooth-muscle dependent impairment; whereas, a greater response to endothelial-independent reagents would imply endothelial-based impairment. Optimum doses were empirically determined in unoperated mice to elicit a maximum vasodilatory or vasoconstrictive response with the lowest concentrations of respective reagents. These doses were then used to assess the reactivity of arterialized capillaries and pre-existing collaterals in operated animals.

Endothelial Dependent and Independent Pathways

To determine whether endothelial-dependent and/or endothelial-independent pathways are responsible for impaired vasodilation, the major components of the relevant cascades first need to be understood. Endothelial-dependent vasodilation is elicited by activating a G-protein coupled receptor (GPCR) cascade that stimulates endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO) and stimulates the endothelium to generate more endothelium-derived hyperpolarizing factors (EDHFs), such as epoxyeicosatrienoic acid (EET), an arachidonic acid metabolite (6, 7, 19, 40). NO binds to smooth muscle cell guanylyl cyclase receptors, which increases cyclic guanosine monophosphate (cGMP) production, thereby activating protein kinase G (PKG). PKG phosphorylation inhibits myosin light chain kinase and reduces intracellular calcium by decreasing the open probability of the calcium channels, ultimately inhibiting actin-myosin cross-bridge cycling to relax the smooth muscle and cause vasodilation (5, 40,

75). Acetylcholine (ACh) can be applied directly over vessels and tissue to initiate the endothelial-dependent vasodilation cascade via GPCR cascade activation and also through hyperpolarizing effects (6, 36). Alternatively, sodium nitroprusside (SNP) breaks down into NO and leads to vasodilation by targeting receptors directly on the smooth muscle and bypassing the endothelium.

Increasing the open probability of voltage-gated potassium channels, another action of EDHFs, also leads to hyperpolarization independent of the endothelium-dependent GPCR cascade (24). Hydrogen sulfide (H_2S) can be applied in the form of sodium hydrogen sulfide (NaHS) to initiate hyperpolarization for endothelial-independent vasodilation. The hyperpolarization closes voltage-gated calcium channels, which prevents influx of calcium and, thereby, decreases intracellular calcium levels. Less calcium is available to bind to calmodulin to create the calcium-calmodulin complex that would otherwise activate myosin light chain kinase (MLCK). Since less MLCK is activated and there is less intracellular calcium for cross-bridge cycling, the smooth muscle relaxes.

Another way to relax the smooth muscle is through prostacyclin production in the endothelium, which can be mimicked with the application of papaverine, a phosphodiesterase inhibitor. Prostacyclin activates adenylyl cyclase (AC) by binding to prostaglandin I_2 (IP) receptors and activating G-protein coupled receptors specific to AC. AC, then, leads to the elevation of cyclic adenosine monophosphate (cAMP) levels and activating protein kinase A (PKA) (14, 38, 40). PKA phosphorylates and inhibits MLCK, thereby relaxing the vascular smooth muscle by decreasing intracellular calcium levels and inhibiting contraction. The consequent reduction of cross-bridge cycling relaxes the

muscle to dilate the vessel. Phosphodiesterase inhibitors like papaverine mimic prostacyclin production by preventing cAMP hydrolysis and degradation to increase cAMP levels (14).

To evaluate the ability for SMCs to dilate, we can apply endothelial-derived relaxing factors (EDRFs), such as nitric oxide, or compounds that mimic EDRFs to stimulate a particular pathway (7). When nitric oxide (NO) compounds are directly applied, the smooth muscle relaxes through direct activation of soluble guanylyl cyclase (**Figure 3**). Guanylyl cyclase increases cGMP to phosphorylate PKG, inhibit MLCK, and inhibit actin-myosin cross-bridge cycling, allowing the smooth muscle to relax.

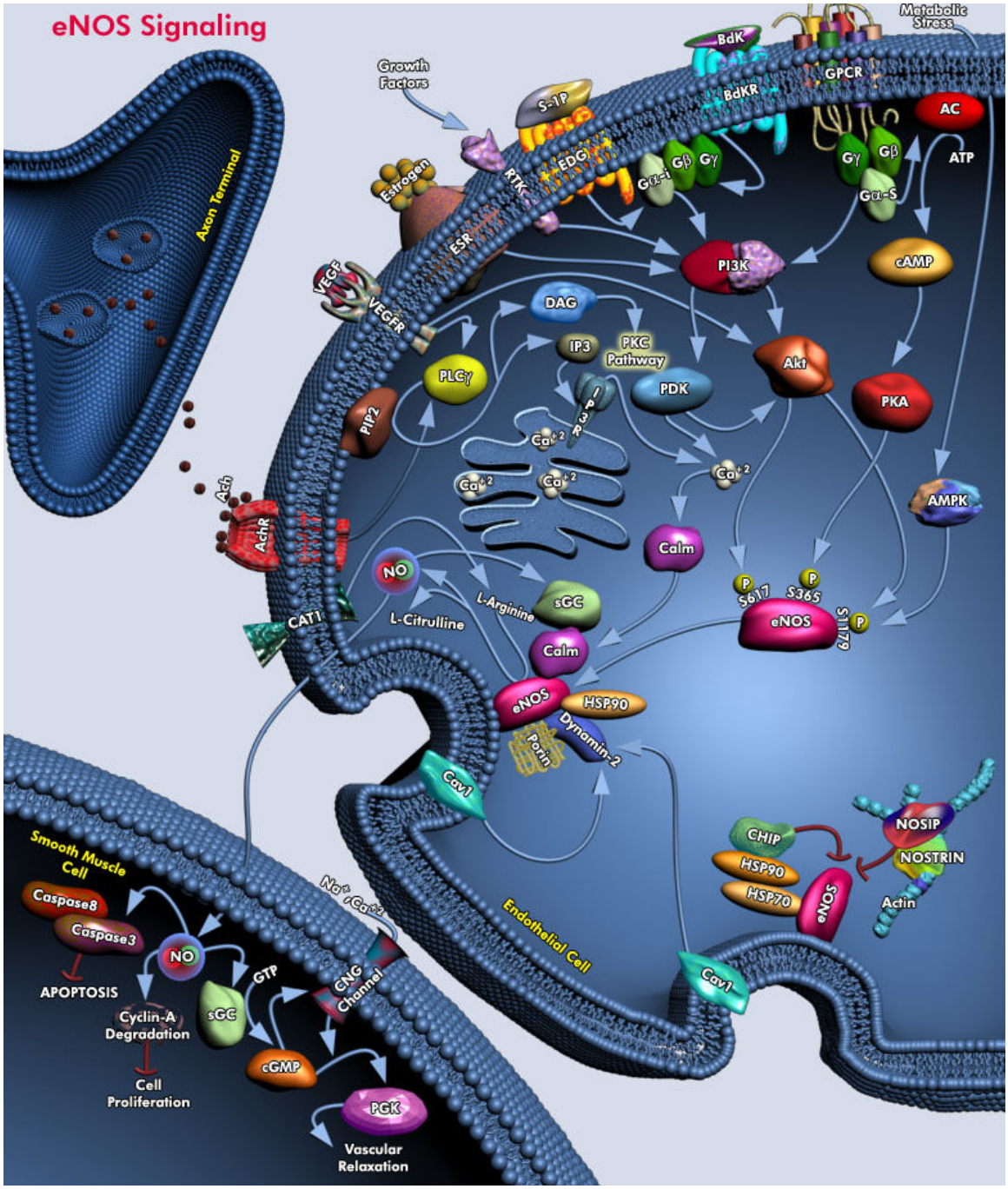

Figure 3. Cell signaling in smooth muscle relaxation (53)**.** With many potential initial stimuli, vascular smooth muscle ultimately dilates in response to nitric oxide (NO).

In addition to analyzing vasodilation, it is valuable to investigate the ability of vessels to vasoconstrict, as both actions are critical to properly regulating tissue perfusion (5). Norepinephrine (NE) is a neurotransmitter that activates alpha-1 adrenergic receptors

on the smooth muscle cells to increase intracellular calcium and constrict the vessel (**Figure 4**) (40).

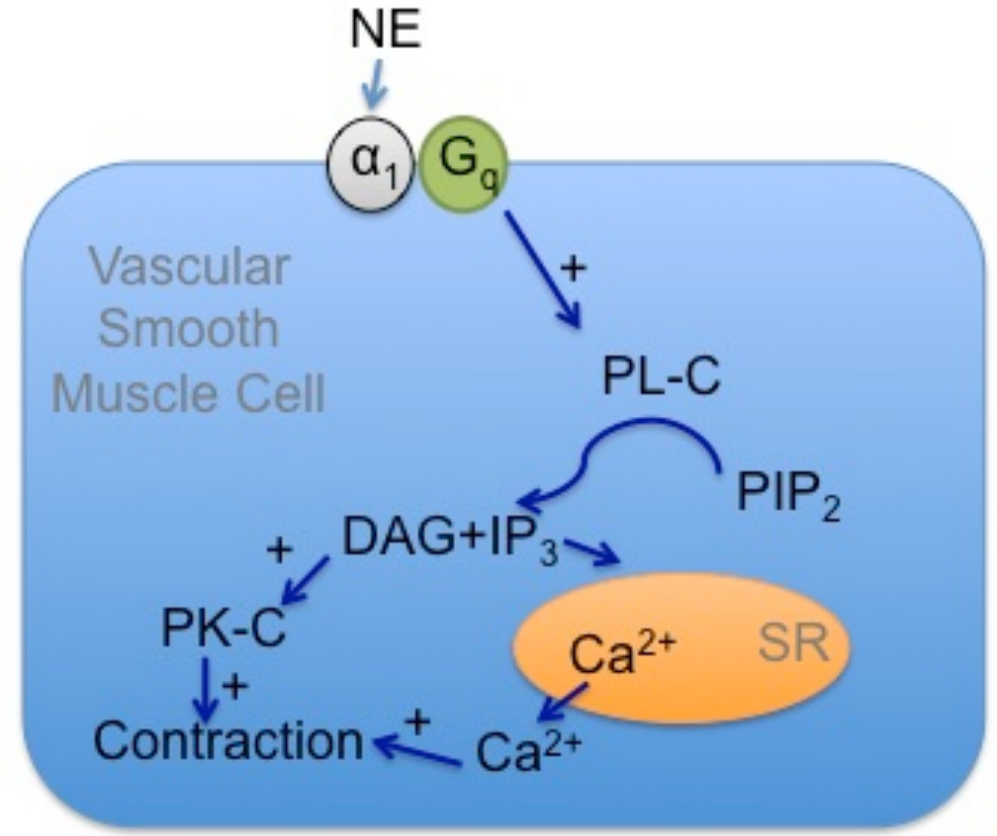

Figure 4. Cell signaling for smooth muscle contraction. Increasing intracellular calcium within the smooth muscle allows for increased cross-bridge cycling.

The adrenergic receptors activate phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases intracellular calcium by binding to and opening the IP_3-sensitive receptor on the endoplasmic reticulum. Increased intracellular calcium increases cross-bridge cycling in the smooth muscle and, thus, causes vasoconstriction.

To confirm a generalized smooth muscle-based vasodilation impairment in immature arterialized collateral capillaries (ACCs), we exposed ACCs to endothelial-

dependent (ACh) and endothelial-independent (SNP) vasodilators, prostacyclin-mimicking (papaverine) and hyperpolarizing (sodium hydrogen sulfide) reagents in a physiological salt solution seven days post-ligation. The effects of the latter two reagents were assessed using sodium nitroprusside (SNP) as a control. In these ways, we tested the hypothesis that smooth muscle-dependent reactivity, including both the hyperpolarization and prostacyclin pathways of vasodilation, would be impaired within the vascular smooth muscle of arterialized capillaries as compared to that of terminal arterioles with equivalent baseline diameters, but that they will still significantly dilate. Impairment specific to the smooth muscle would materialize as equal vessel impairment in response to endothelial-independent reagents, as compared to the response to endothelial-dependent reagents.

METHODS

Animal Care and Housing

Male Balb/C mice were housed in microisolator cages within temperature-controlled rooms in the University Vivarium for the duration of the study on a 12-hour light/dark cycle. Male mice are used consistently to avoid variation in restoration capabilities due to genetic differences between genders, such as higher baseline VEGF and eNOS levels in females (49). All mice were provided food and water ad libitum, 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 protocols that were approved by the Cal Poly State University SLO Institutional Animal Care and Use Committee.

Vascular Reactivity with Intravital Microscopy – Dose Response

To determine the optimum concentrations where there is a consistent response to ACh, SNP, NE, papaverine, and NaHS, dose response studies were performed in which increasing concentrations were applied for each reagent until additional doses did not result in an increase in vessel diameter. Evenly distributed doses by factors of ten were used because the vessels tend to respond in a sigmoid pattern such that there is consistent, low reactivity with the lower doses at the left of the curve, highly sensitive and exponential increases at the center, and a consistent plateau at the right with high reactivity in response to the higher doses. Vasoactive agents were delivered to the spinotrapezius preparation in a physiological salt solution (PSS), which was prepared daily and contained (in mM) 131.9 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 2 $CaCl_2$, and 18 $NaHCO_3$. PSS was heated to 45 ºC, bubbled with 5% CO_2-95% N_2, maintained at a pH of

~7.4, and flowed over the preparation at 35 ºC and ~2 $mL \cdot min^{-1}$ to most closely replicate anaerobic and physiologic conditions.

Each mouse was initially anesthetized with 4-5% isoflurane in oxygen in an induction chamber before being transferred to a preparation bench where the isoflurane was reduced to ~1-3% and maintained at ~0.5-1.0 $L \cdot min^{-1}$ via nose cone throughout the duration of the procedure. The hair on the anterior dorsal aspect of each mouse was removed with trimming clippers and depilatory cream. Following skin preparation, mice were transferred to a heat pad (CWE Inc., 08-13000) in the prone position.

Internal temperatures were maintained at 35º C via heat pad controlled by a rectal thermistor. Following an initial skin incision at the caudal end of the spinotrapezius, the skin was retracted and superficial fascia gently removed to expose the muscle. The exposed tissue was continually irrigated with PSS, 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 after a 30-minute stabilization period. Vasoactive agents were administered to the PSS at 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M for SNP, ACh, and NE, at 10^{-6}, 10^{-5}, 10^{-4}, $10^{-3.5}$, and 10^{-3} M for NaHS, and at 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M for papaverine. Videos of vessel diameter were captured during the final minute of a 5 to 15-minute waiting period and the procedure was repeated on the contralateral limb.

Spinotrapezius Lateral Feed Artery Ligation

To stimulate the arterialization of collateral capillaries, the lateral spinotrapezius feed artery was ligated (**Figure 5**). Mice were anesthetized and prepared as described above. Buprenorphine analgesic (0.075 $mg \cdot kg^{-1}$) was subcutaneously administered and veterinary ophthalmic ointment was applied to the eyes of the mice to prevent corneal desiccation. A skin incision was made above the cranial, lateral edge of the spinotrapezius where it intersects with the fat pad. Sterile 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.

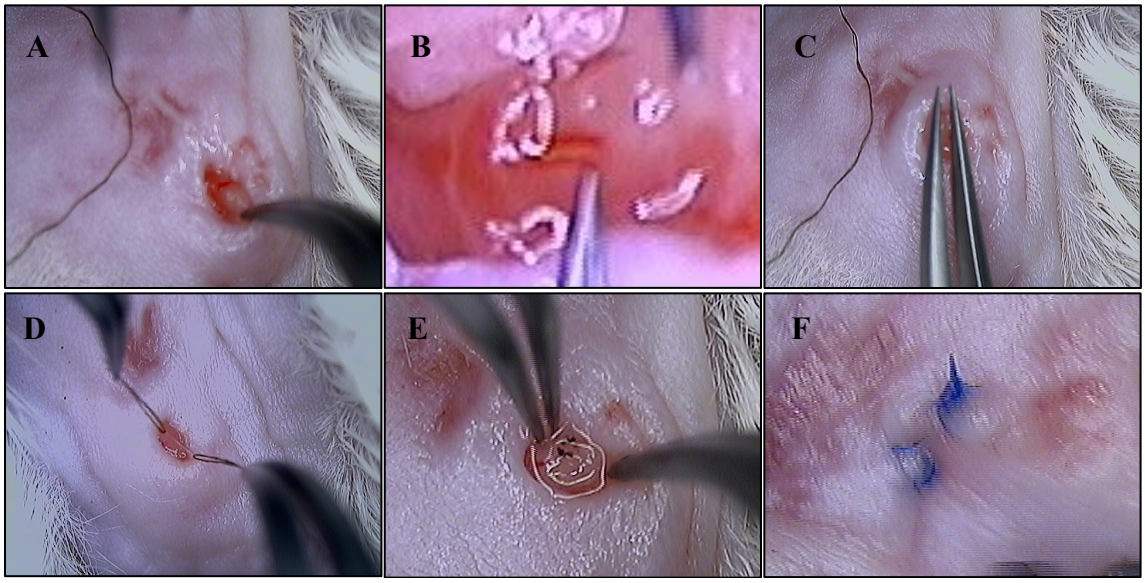

Figure 5. Spinotrapezius ligation surgery. A) The initial skin incision is made and the lateral edge of the spinotrapezius muscle is exposed via blunt dissection. **B)** The artery vein pair is exposed and **(C)** separated. **D)** The artery is isolated with free strands of silk suture and **(E)** ligated. **F)** The incision is closed with 7.0 prolene suture.

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 an incision was made on the contralateral, or sham, side. The lateral edge of

the spinotrapezius was exposed, the area was blunt dissected, and the incision was closed with suture. Buprenorphine 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 $mg \cdot mL^{-1}$) mixed in with the water.

Vascular Reactivity with Intravital Microscopy

Seven days following each ligation surgery the spinotrapezius muscle was re-exposed. The same protocol was followed as described above, but each vasodilator agent was only administered to the area once at 10^{-5} Molar (ACh, SNP, NE), 10^{-4} Molar (papaverine), or 10^{-3} Molar (NaHS) concentrations. Arterialized capillaries or pre-existing collaterals were analyzed on the ligated side and equivalently-sized contralateral arterioles were analyzed on the sham side. Following the procedure, both the ligated and sham spinotrapezius muscles were also fixed in situ with topical application of 4% paraformaldehyde (pfa), removed, post-fixed overnight in 4% pfa, and stored in PBS at 4º C.

Imaging and Statistical Analysis

The images/videos were analyzed using AVA software to compare diameters before and after reagent application in the sham and arterialized collateral capillaries or pre-existing collaterals. Differences in resting and dilated/constricted diameters, and percent changes between control and ligated sides were evaluated by homoscedastic t-tests and one-way ANOVA. Data are presented as averages ± standard error.

RESULTS

The purpose of this study was to test the hypothesis that a generalized smooth muscle-based vasodilation impairment is present seven days following upstream arterial occlusion. Before testing this hypothesis, optimum concentrations were determined for the endothelial-dependent vasodilator, ACh (**Figure 6**), for the endothelial-independent

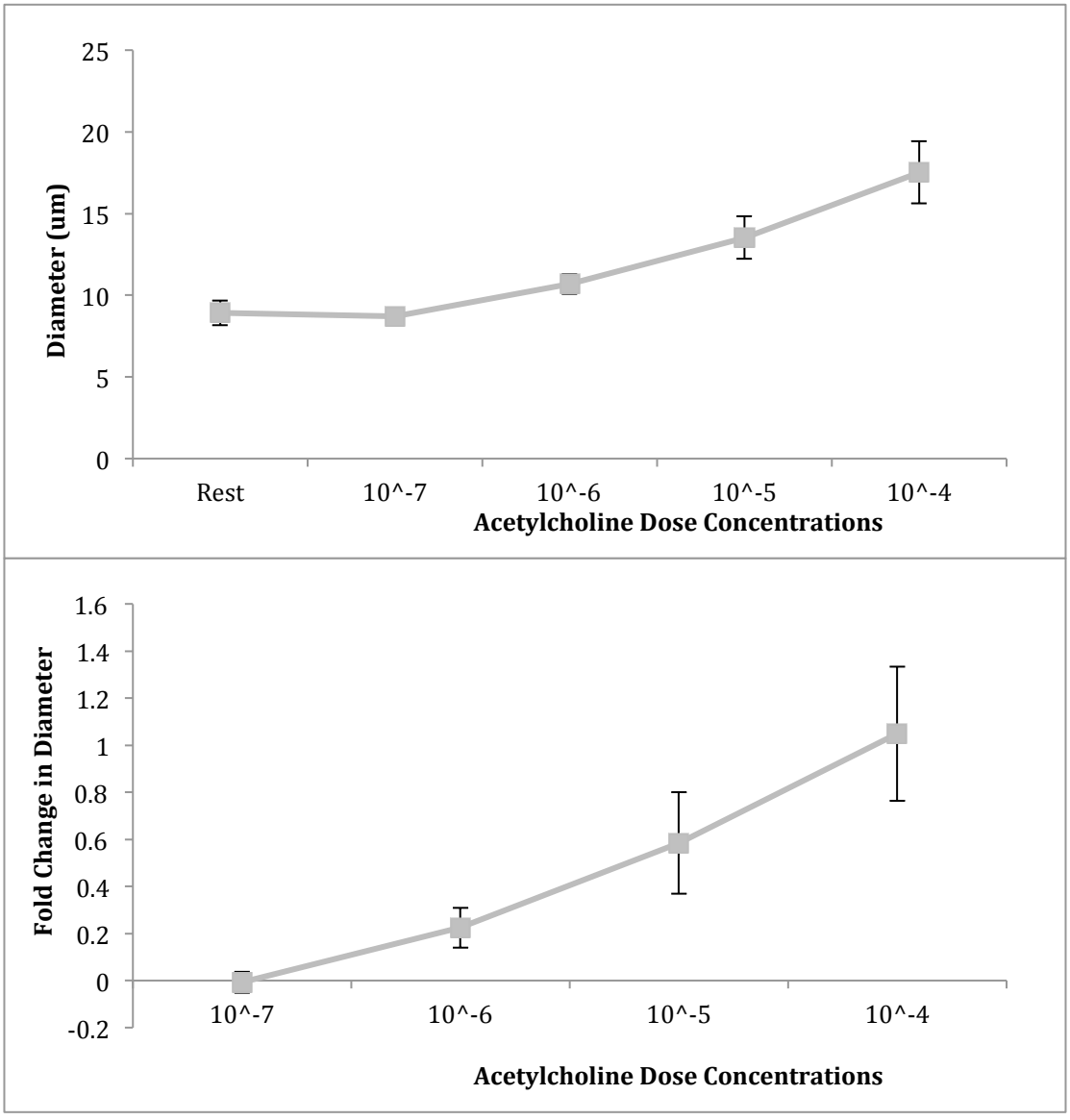

Figure 6. Endothelial dilator dosage response curves in unoperated animals. A) Terminal arteriole diameters (μm) pre and post exposure to 10-fold dilutions of ACh (n=7). **B)** Percent changes post ACh exposure.

vasodilators, SNP, papaverine, and NaHS (**Figure 7**), and for the endothelial-independent vasoconstrictor, NE (**Figure 8)**.

Vessels responded in a roughly sigmoid pattern to all vasoactive agents, such that maximal or near maximal vasodilation or vasoconstriction was attained where the sigmoid curves begin to plateau. Optimal concentrations were determined according to

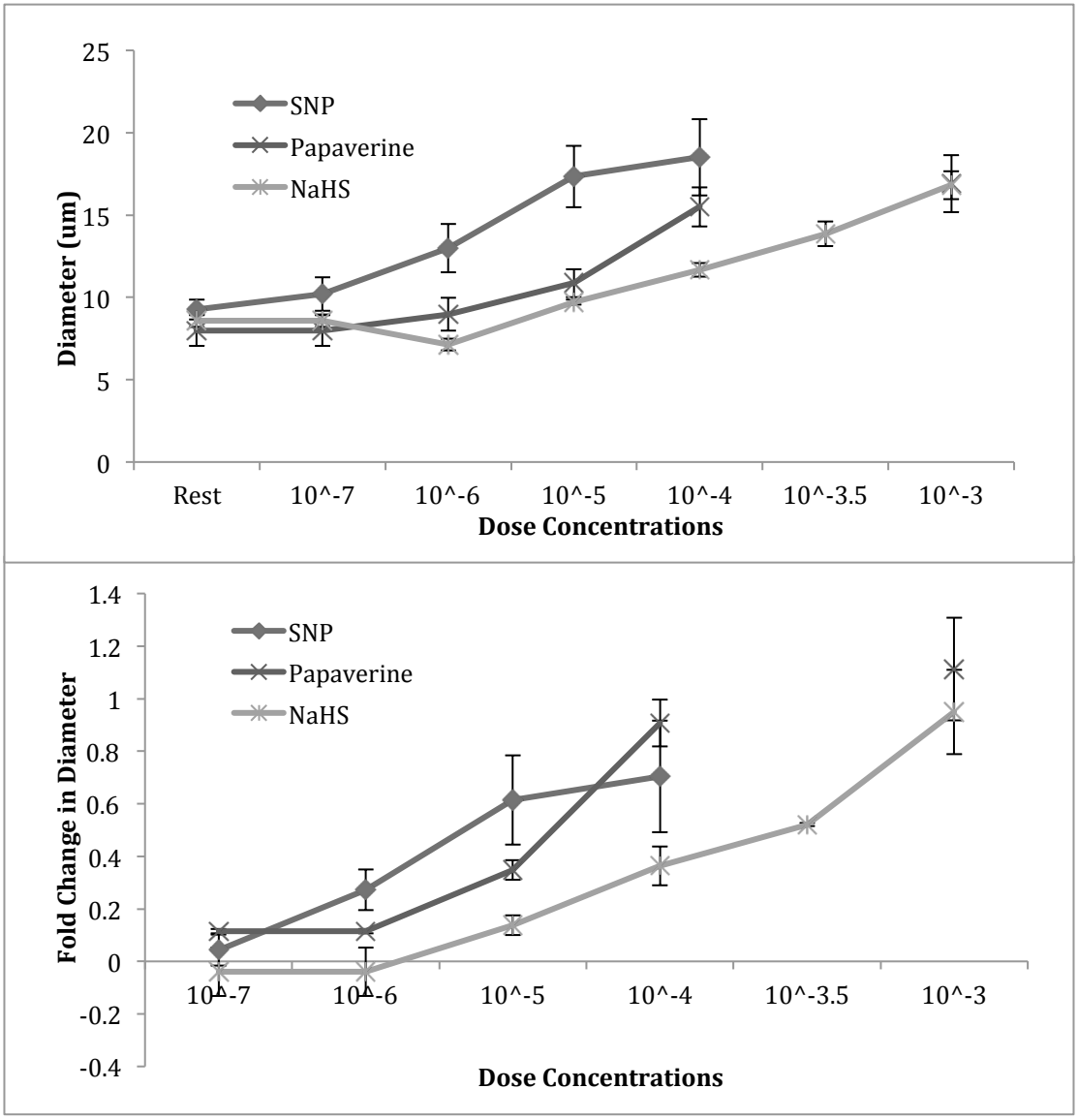

Figure 7. Smooth muscle dilator dosage response curves in unoperated animals. **A)** Terminal arteriole diameters (μm) pre and post exposure to 10-fold dilutions of SNP (n=9), papaverine (n=6), and NaHS (n=7). **B)** Percent changes following SNP, papaverine, and NaHS dilution exposure.

the curves, relative responses in comparison to other reagents, and lab chemical safety to be 10^{-5} M in response to SNP (9.5 ± 0.5 μm vs. 17.5 ± 5.5 μm) and NE (13.5 ± 4.5 μm vs. 6.5 ± 1.5 μm), 10^{-5} M for ACh (9 ± 1 μm vs. 17.5 ± 5 μm) and papaverine (8 ± 1 μm vs. 15.5 ± 1 μm), and 10^{-3} M for NaHS (8.5 ± 0.5 μm vs. 17 ± 1 μm).

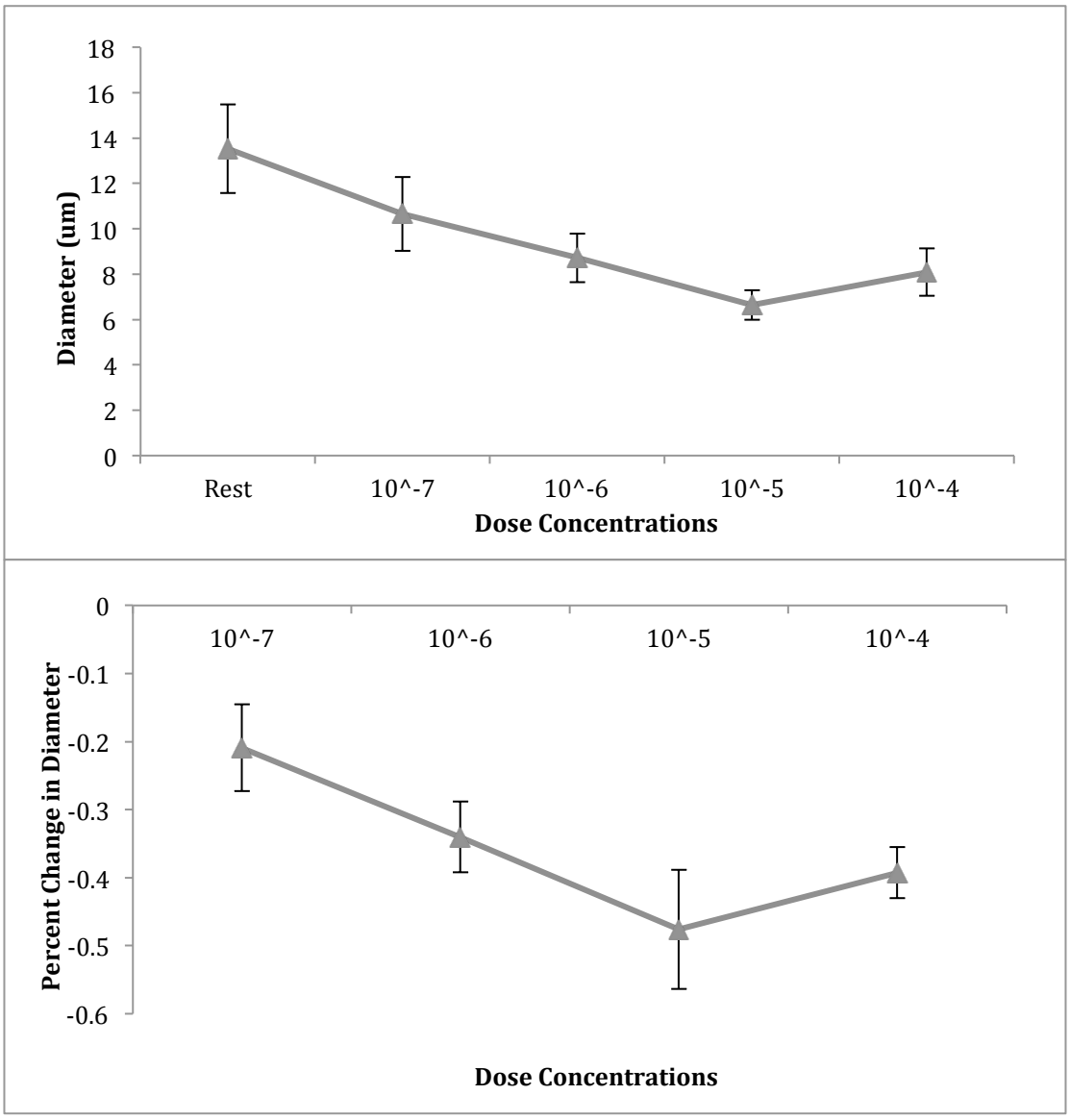

Figure 8. Vasoconstrictor dosage response curves in unoperated animals. A) Arteriole diameters (μm) pre and post exposure to 10-fold dilutions of NE (n=5). **B)** Percent changes post NE exposure.

Using the optimal concentrations calculated from the dose responses, I tested the hypothesis that the absence of functional vasodilation at day-7 following feed artery ligation is due to smooth muscle cell dysfunction. Arterialized collateral capillaries (ACCs), pre-existing collaterals, and equivalently sized contralateral arterioles were exposed to vasoactive reagents seven days following spinotrapezius feed artery ligation. Both the sham and ligated sides were exposed to an endothelial-dependent vasodilator, acetylcholine (ACh), an endothelial-independent vasodilator, sodium nitroprusside (SNP), and an endothelial-independent vasoconstrictor, norepinephrine (NE) via superfusion (**Figures 9, 10**).

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; however, the terminal arterioles dilated significantly more than did the arterialized capillaries (**Figure 9**). In the ACCs, ACh dilated from 8.5 ± 0.5μm to 10 ± 0.5 μm, while SNP dilated from 8.5 ± 0.5 μm to 10.5 ± 0.5 μm. NE (endothelial-independent) failed to significantly constrict the ACCs from 9 ± 1 μm to 8 ± 1 μm. In the sham arterioles, ACh dilated from 8.0 ± 0 μm to 14 ± 1.5 μm and SNP dilated from 8 ± 0 μm to 15.5 ± 1 μm. NE decreased vessel diameters in the sham to a point at which luminal diameter was absent (13.5 ± 2 μm to 0 ± 0 μm) so that the terminal arterioles constricted significantly more than did the arterialized capillaries. In comparing the sham arterioles to ACCs, percent changes in diameter were less in response to ACh (69.5 ± 14 μm vs. 19 ± 3.5 μm), SNP (95.5 ± 9 μm vs. 20.5 ± 5 μm), and NE (-95.5 ± 4.5 μm vs. -16.5 ± 11.5 μm). The responses to ACh (endothelial-dependent) and SNP (endothelial-independent) were similar within the respective vessels.

Reactivity in the PECs was similar to that in the ACCs at day seven (**Figure 10**) as PECs trended towards less vasodilation in response to both ACh and SNP. NE (endothelial-independent) significantly decreased vessel diameters in the sham and ligated sides, again, to approximately the same degree. In the PECs, ACh dilated from 9 ± 2.5 μm to 34 ± 3 μm, SNP dilated from 17.5 ± 2 μm to 34.5 ± 3 μm, and NE constricted from 24 ± 3 μm to 7 ± 2 μm. In the sham arterioles, ACh dilated from 15.5 ± 2.5 μm to 30 ± 3.5 μm, SNP dilated from 15.5 ± 3 μm to 35 ± 4.5 μm, and NE constricted from 25 ± 3.5 μm to 11.5 ± 1.5 μm. Comparing the percent changes in diameter for sham and ligated, the responses were similar to ACh (100.5 ± 32.5 % vs. 101 ± 33.5 %) and NE (-47.5 ± 16 % vs. -67 ± 9 %), but with an impaired response to SNP (145 ± 26.5 % vs. 98 ± 19.5 %). Because the vessels were not always allowed to return to their resting diameters prior to applying NE, the baseline values are higher for NE than they are for ACh and SNP.

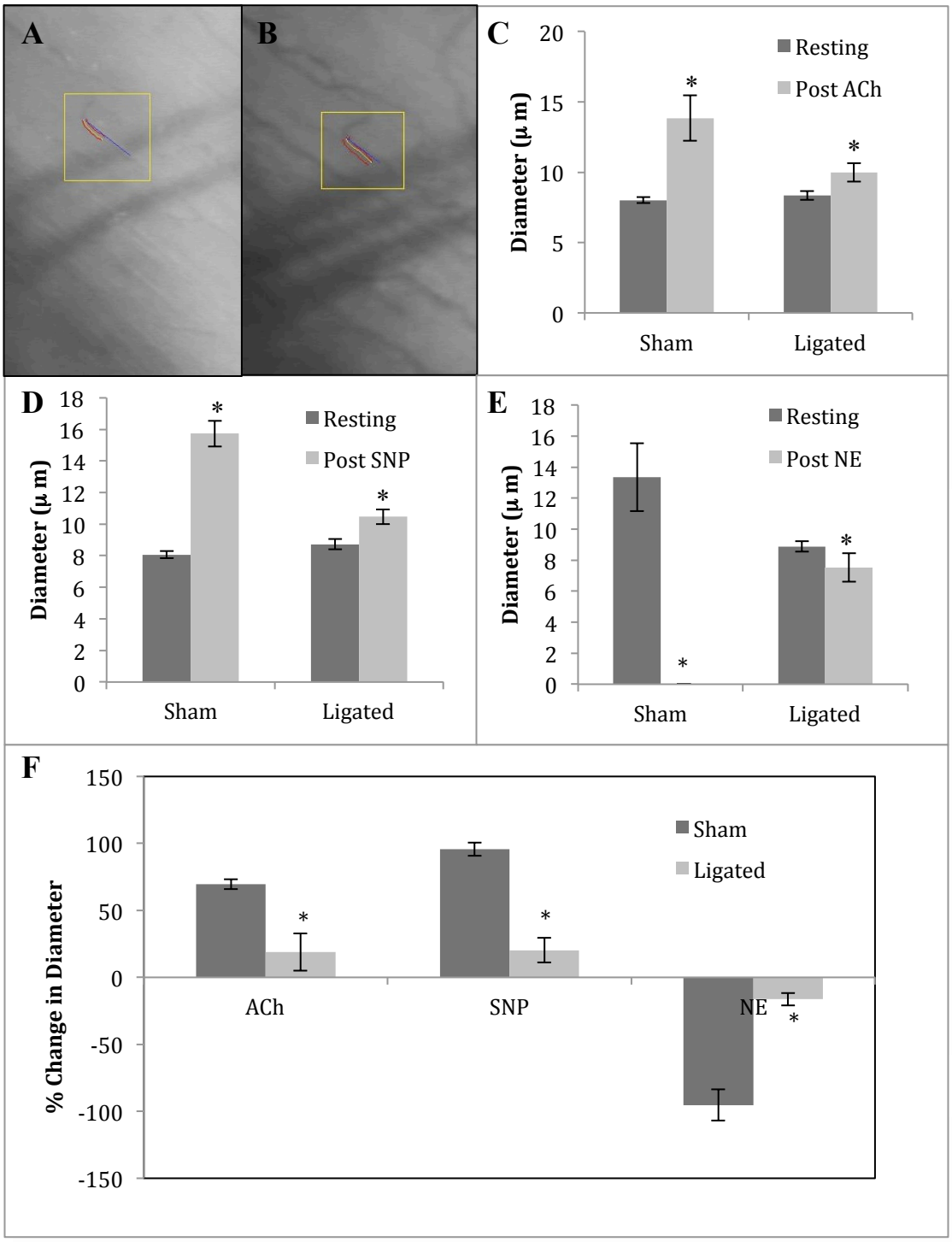

Figure 9. Vascular reactivity in arterialized capillaries at day 7. Arterialized capillaries pre **(A)** and post **(B)** exposure to 10^{-5} M SNP, visualized with intravital microscopy. Diameters (µm) pre and post exposure to 10^{-5} ACh (n=7) **(C)**, 10^{-5} M SNP (n=7) **(D)**, and 10^{-5} M NE (n=6) **(E)**. **F)** Percent changes of vessel diameter in response to ACh, SNP, and NE**; *** indicates $p < .05$ using a paired student's t-test.

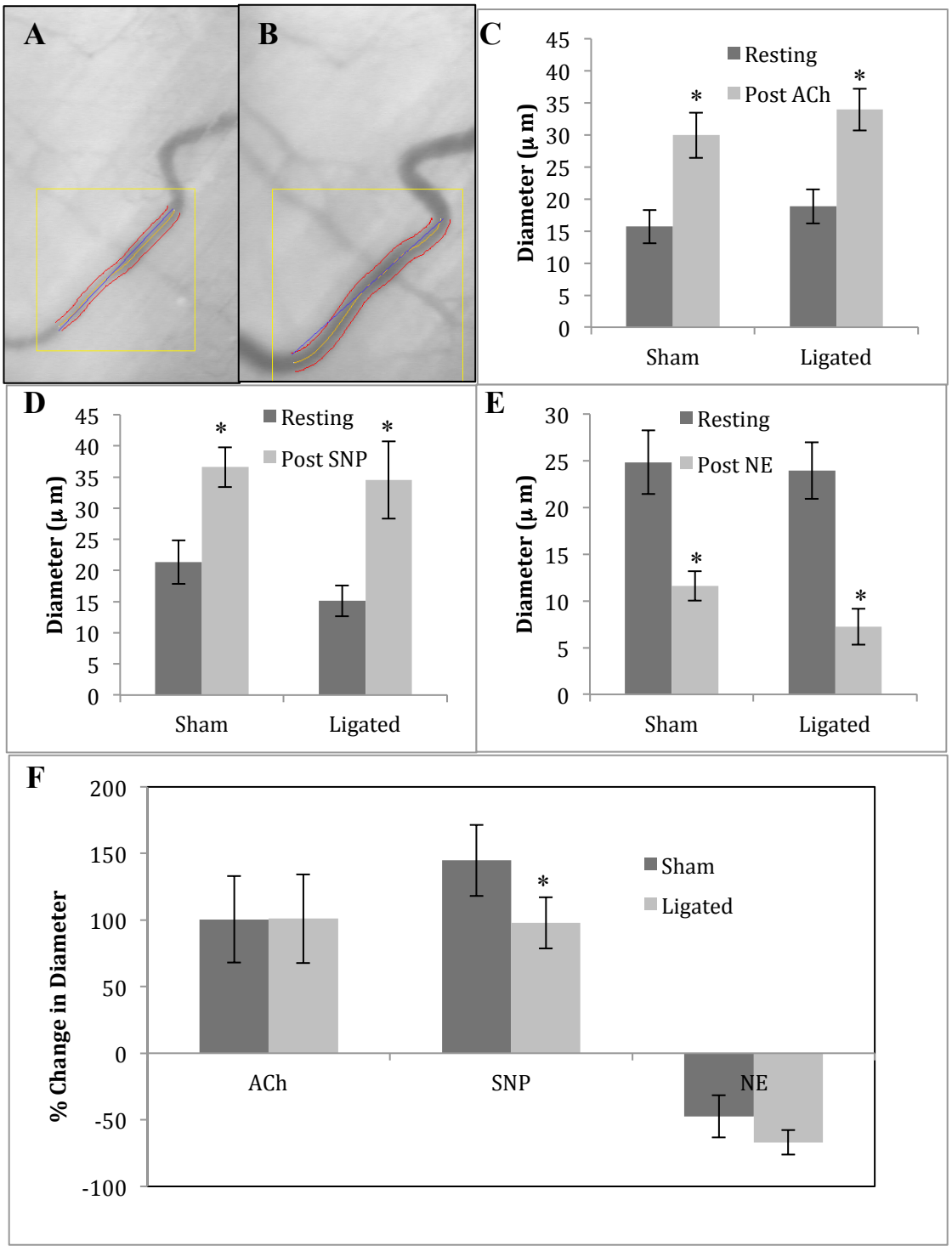

Figure 10. Vascular reactivity in pre-existing collaterals (PECs) at day 7. PECs pre **(A)** and post **(B)** exposure to 10^{-5} M ACh, visualized with intravital microscopy. Diameters (μm) pre and post exposure to 10^{-5} ACh **(C)**, 10^{-5} M SNP **(D)**, and 10^{-5} M NE (n=7 ligated, n=4 sham) **(E)**. **F)** Percent changes of vessel diameter in response to ACh, SNP, and NE**;** * indicates $p < .05$ using a homoscedastic t-test.

With many pathways available for vasodilation within the smooth muscle, there are many possible sources for impaired reactivity. To identify which vasodilation pathways, in particular, are impaired within the smooth muscle cells of immature collaterals, arterialized collateral capillaries, pre-existing collaterals, and equivalently sized contralateral arterioles were exposed to additional endothelial-independent vasodilators seven days following spinotrapezius feed artery ligation. Both the sham and ligated sides were exposed to the phosphodiesterase inhibitor, papaverine, and to the hyperpolarizer, sodium hydrogen sulfide (NaHS), via superfusion.

Following the same pattern as ACh and SNP, both papaverine and NaHS significantly vasodilated arterialized collateral capillaries (ACCs) and terminal arterioles (**Figure 11**). In the ACCs, papaverine dilated from 8.5 ± 0.5 μm to 11 ± 0.5 μm, and NaHS dilated from 8.5 ± 0.5 μm to 12 ± 1 μm. In the sham arterioles, papaverine dilated from 8.5 ± 0.5 μm to 18.5 ± 2.5 μm, and NaHS dilated from 8 ± 0.5 μm to 19.5 ± 2.5 μm. Though the ACCs dilated significantly from rest, the ACC percent increases were reduced when compared against the terminal arterioles in response to papaverine (32 ± 5.5 % vs. 97 ± 16 %) and NaHS (40 ± 4.5 % vs. 86.5 ± 6 %).

Interestingly, while SNP, papaverine, and NaHS all significantly increased vessel diameters in the arterioles of the sham side and PECs in the ligated side to a similar degree, the PECs only trended towards a lesser vasodilation in response to each reagent when compared to the sham (**Figure 12**). In the PECs, papaverine dilated from 32 ± 8 μm to 49 ± 9 μm, and NaHS dilated from 31.5 ± 7 μm to 49.5 ± 9 μm. In the sham arterioles, papaverine dilated from 23.5 ± 1 μm to 54 ± 9 μm, and NaHS dilated from 25.5 ± 2.5 μm to 56 ± 8 μm. PEC percent increases trended towards impairment when compared against

the contralateral arterioles in response to papaverine (59 ± 12 % vs. 127.5 ± 35 %) and NaHS (61.5 ± 10 % vs. 122.5 ± 37 %).

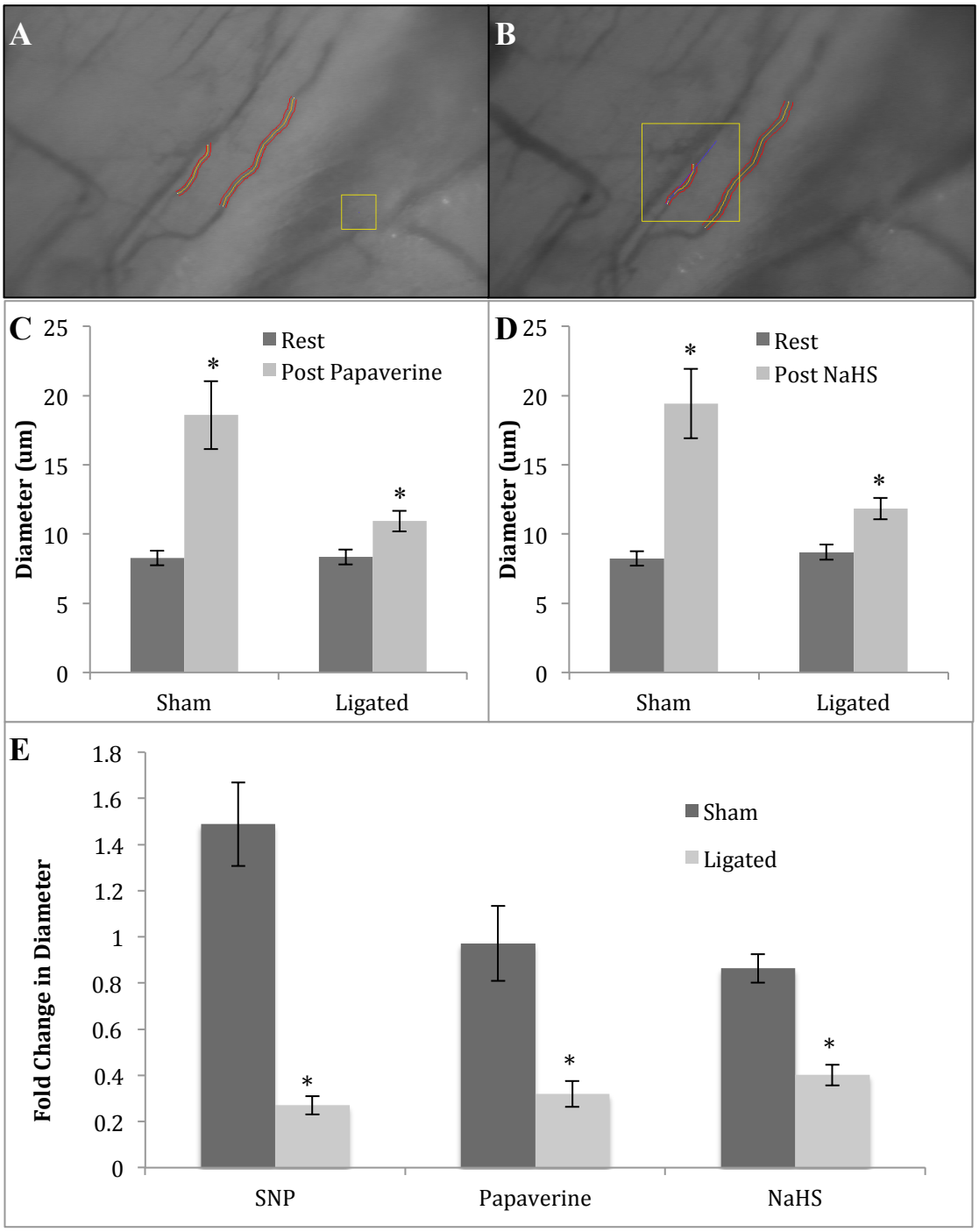

Figure 11. Modes of impaired SMC-based vasodilation in arterialized capillaries. Representative images pre **(A)** and post **(B)** exposure to 10^{-5} M SNP, visualized with intravital microscopy. Diameters (μm) pre and post exposure to 10^{-5} M papaverine (n=7) **(C)**, and $10^{-3.5}$ M NaHS (n=6) **(D)**. **E)** Percent changes of vessel diameter in response to ACh, SNP, and NE**;** * indicates $p < .05$ using a homoscedastic t-test.

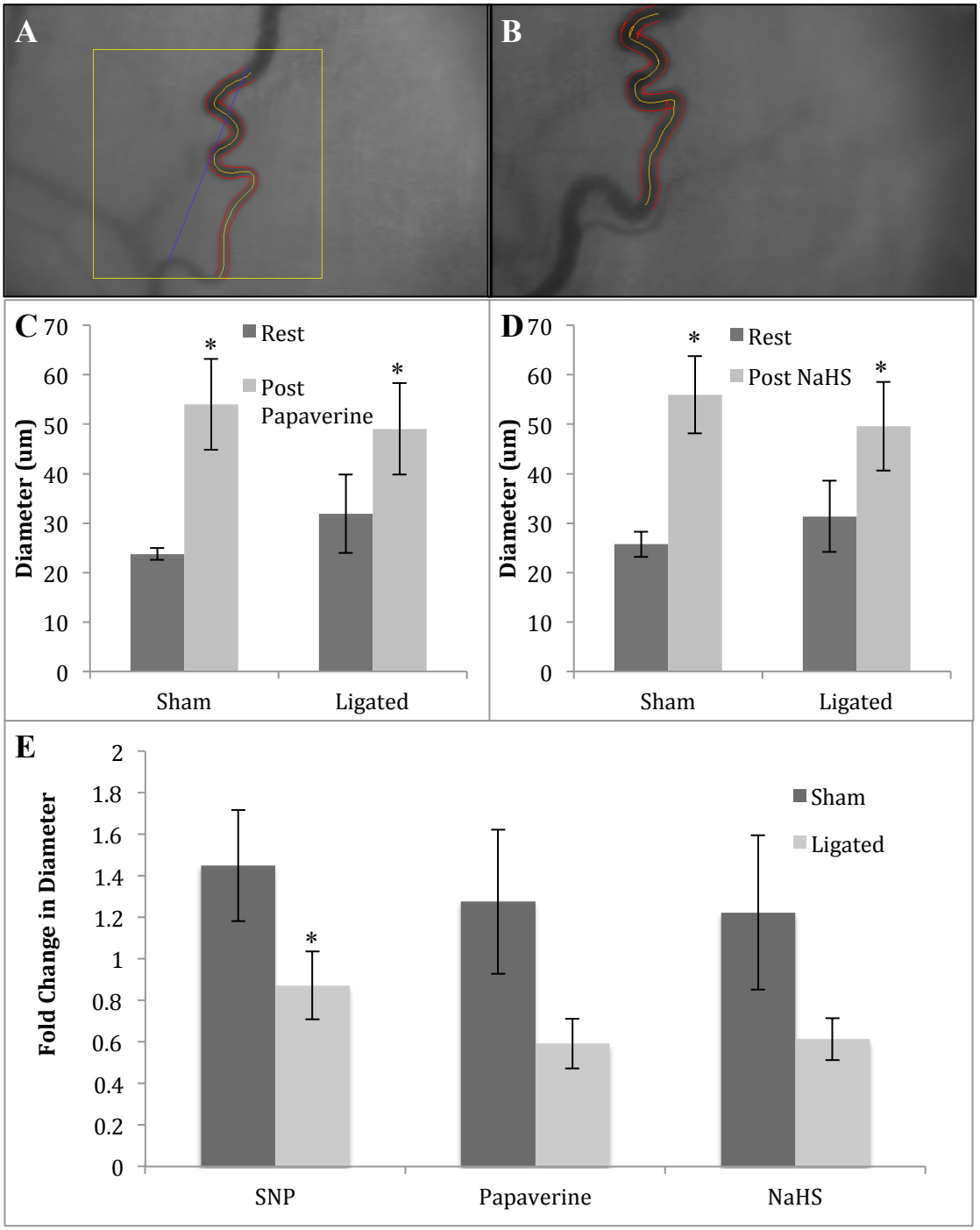

Figure 12. Pre-existing collateral SMC-based vasodilation at day seven. Representative images pre **(A)** and post **(B)** stimulation. Exposure to 10^{-5} M SNP visualized with intravital microscopy. Diameters (μm) pre and post exposure to 10^{-4} papaverine (n=3) **(C)** and 10^{-3} M NaHS (n=3) **(D)**. **E)** Percent changes of vessel diameter in response to SNP control (n=7), papaverine, and NaHS**;** * indicates $p < .05$ from rest using a homoscedastic t-test.

DISCUSSION

Vasodilation is absent in arterialized collateral capillaries in response to electrode-induced muscle contraction seven days following an occlusive event (13). To investigate the source of impairment, we tested the hypothesis that the dysfunction is smooth muscle-dependent by assessing vasodilation in response to various endothelial-dependent (ACh) and endothelial-independent agents (NE, SNP, papaverine, NaHS). Because we found that the relative impairment in vasodilation did not vary between the vasodilators within the arterialized collateral capillaries (ACCs), it is reasonable to conclude that this impairment is due to smooth muscle cell dysfunction. Impaired reactivity in ACCs at seven days post-ligation could be explained in several ways, including smooth muscle cell phenotype, ECM remodeling, and lack of innervation. Endothelial cell impairment may contribute to the impaired vasodilation in the arterialized capillaries, as the functionality of both cell types is altered during arteriole remodeling; however, we cannot associate the impairment with dysfunctional endothelial cells because the necessary testing was not included in the study (17, 39, 45).

At seven days, ACCs failed to dilate in response to a functional stimulation; however, they did significantly dilate in response to ACh, SNP, papaverine, and NaHS (13). This discrepancy can be explained by a more targeted vasodilation from the local application of dilators as compared to the electrode-induced muscle contraction. Local signals to dilate or constrict are typically conducted across the endothelial cells to change the diameter of upstream arterioles and effectively increase regional blood flow in response to a local stimulus (17, 31). Because they do not typically dilate or constrict, capillaries may not normally conduct these signals so that, even when the capillaries have

arterialized, they may not yet have the capacity to communicate the vasodilation signal stimuli at day seven (17). Although the impaired vasoconstriction is likely due to reduced smooth muscle cell function, it may also be explained by a lack of innervation of the arterialized capillaries, which could limit contractile abilities (26, 69).

In addition to pharmacological evaluation of arterialized collateral capillaries (ACCs), we also sought to gain insight into the nature of impaired vasodilation of ACCs independent from genetic variation between strains by evaluating pre-existing collaterals (PECs), a more studied collateral vessel form (11, 43, 79). Because the smooth-muscle layer is already present within the PECs at the time of ligation, we anticipated that the vessels would undergo a less dramatic remodeling than the ACCs, and, therefore, provide insight into the role of cell rearrangement and proliferation versus cell recruitment as the primary impetus for smooth muscle-based dysfunction (11, 79). The anatomical differences in size and layout may also contribute to vessel function through varied shear stress, as the PECs tend to be large, tortuous, and singular, while ACCs tend to be smaller, less tortuous, and more numerous.

In a similar manner to the ACCs, PECs trended towards impaired vasodilation in response to the smooth muscle-based dilators (SNP, papaverine, and NaHS) at day-seven. Differently from the ACCs, however, the PECs had no impairment in response to the endothelial-dependent dilator (ACh) and endothelial-independent constrictor (NE). The likely explanations for smooth muscle impairment in PECs are similar to those for the ACCs, with the additional explanation of cell proliferation in arteriogenesis and without the possibility of a lack of innervation. It is also possible that there were too few replicates to accurately represent PEC reactivity, or that impairment in the PECs is

specific to the NO and prostaglandin pathways, with the endothelium-derived hyperpolarizing factor (EDHF) pathway and electrical signaling relatively intact.

Smooth Muscle Cell Phenotype

Although ACC dilation is possible seven days post-occlusion, it is severely reduced. It is likely that the development and recruitment of the smooth muscle cells (SMCs) contributes to this impairment because the cells are still remodeling at day-seven. Capillaries do not normally have smooth muscle cells as a part of their structure; however, the increased flow in the ischemic tree may stimulate an enhanced function of blood flow regulation via smooth muscle. The perivascular cells surrounding capillaries only stain positive for smooth muscle α-actin after they arterialize so that any present smooth muscle cells have either recently migrated to the area or recently differentiated, implying a potential need for an adjustment period (25). Perivascular cells can be recruited from nearby arterioles or from the immediately surrounding tissue to provide the capillaries with a smooth muscle layer (48, 68). Regardless of their origin, the cells will take time to arrange and may need time to mature into contractile SMCs. For example, if fibroblasts are developing into SMCs, they may not yet have sufficient levels of actin and myosin to properly contract or relax. Thus, the immaturity of the smooth muscle within arterialized capillaries would impair vascular reactivity. An immature phenotype could be identified through gene or protein expression of the vessel wall, or through further pharmacological interrogation of particular vasodilation/vasoconstriction pathways. Other vasodilation pathways can be evaluated via intravital microscopy, such as arachidonic acid synthesis (bradykinin) (46). Though it would not address cell origins, the presence of specific genes or proteins could be analyzed to indicate smooth muscle

phenotypes at the day-seven time-point (8, 25). Immaturity could also be evaluated by histological staining for the prevalence of myosin heavy chain, as the minimally-contractile cells may have less or a varied isoform of myosin heavy chain (34, 57).

Extracellular Matrix Remodeling

An overly stiff or flexible ECM may also contribute to the vessel dysfunction at seven days post-ligation. In addition to the rearrangement of smooth muscle cells, the extracellular matrix-integrin-cytoskeletal axis is activated in response to long-term exposure to increased shear stress, as experienced in arterializing capillaries and developing PECs (36, 42). In the axis, cell-cell and cell-extracellular matrix connections are strengthened to maintain vascular wall structural integrity; it is also dynamically involved in controlling vasoreactivity. In the presence of increased shear stress, integrins bind to ECM proteins to trigger a signal that activates receptor tyrosine kinases on the endothelium to induce vasodilation (32, 44). Proteases and matrix metalloproteinases (MMPs) are also activated to degrade and remodel the ECM and to modulate cell movement and morphogenesis, in attempts to make constriction and/or dilation more efficient (16, 67). If the ECM is still responding to the increased shear stress seven days post-occlusion ECM-cell signaling, vascular wall flexibility, and surrounding cell orientation will not be optimal. Thus, dilation will not be fully efficient and it will be impaired in the early remodeling stages of ACCs and PECs. ECM composition could be identified through staining for collagen fiber density, integrin, and matrix metalloproteinase (MMP) presence. An increase in collagen fiber density and homogeneous cell orientation in the mature ACCs or PECs, for example, may indicate an

ECM that is optimized for vasodilation and constriction, and the opposite may be found in immature vessels.

Lack of Innervation

Although the impaired vasoconstriction observed at day-seven is likely due to generalized smooth-muscle dysfunction, it is possible that a lack of innervation contributes to an initial inability of ACCs to contract. Though sympathetic nervous stimulation can cause dilation through β-adrenergic receptors, it typically causes vasoconstriction in skeletal muscle arterioles by activation of α-adrenergic receptors on the smooth muscle (40, 50). Capillaries are not directly innervated so that, even though adrenergic receptors are expressed in the endothelium, a lack of integration of the ACC receptors into the neurovasculature could be displayed as insensitivity to vasoconstrictors like norepinephrine (NE) (41). As the ACCs develop, they may become innervated by sympathetic nervous system (SNS) fibers and integrate α-adrenergic receptors on the smooth muscle to, then, be more responsive at day 21. Staining the capillary walls and surrounding tissue before and after arterialization in unoperated and day-seven mice, respectively, could identify an initial lack of innervation to explain deficient vasoconstriction capabilities.

Endothelial Communication Disruption

At day seven, functional vasodilation was absent; however, dilation in response to superfused vasodilators was present, though impaired. This discrepancy may be explained by a lack or dysfunction of endothelial cell-cell communication (20). Because the vasodilating reagents were applied directly, they targeted the endothelial and smooth muscle cells within the arterialized capillaries, bypassing any endothelial-endothelial and,

in the case of endothelial-independent reagents, endothelial-smooth muscle communication pathways, to attain vasodilation. On the other hand, the functional protocol relies on the endothelial cell-cell conduction circuit to communicate a dilation signal upstream from the site of increased metabolism due to electrode stimulated muscle contraction. Capillaries may not naturally be included in the endothelial conduction circuit found in arteries and arterioles to propagate dilation/constriction along a vessel, initially lacking functional gap junctions between endothelial cells to communicate a signal within the circuit (2). If the endothelial gap junctions are still developing in the ACCs at day seven, it is possible that they mature with the rest of the vessel by day 21 to function normally.

The incoordination of these junctions may also be explained by the disruption of smooth muscle and endothelial communication as the capillary arterializes. 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), so that the proper formation of endothelial gap junctions is also dependent on proper communication between the endothelium and smooth muscle (39, 70). Ang1, which regulates genes in endothelial cells, is involved in recruiting the SMCs and maturing the vasculature, so that successful vascular development depends on paracrine communication with ECs and also on endocrine factor processing (39). In conjunction with underdeveloped paracrine and endocrine communication, myoendothelial junctions (MEJs) may still be forming between the ECs and SMCs. Vessel reactivity impairment at day-seven, which is

eventually restored at day 21, could be explained by temporary disruption of these heterocellular communications as the capillaries are arterializing.

To evaluate the hypothesis that an interruption of endothelial cell communication is a source of impaired functional vasodilation, application of dilating agents must be isolated to very specific locations on the blood vessels via micropipettes. Dilators such as adenosine and acetylcholine can be applied locally or distant from the site of interest on the vessel, and the dilation response can be measured following each application (17). Endothelial activity and responses can be tracked with changes in calcium ion concentration via fluorescent dyes, along with luminal diameter measurement via intravital microscopy. Reduced activity, in the form of less calcium fluctuation and less diameter increase, in response to distant dilator exposure at seven days post occlusion would indicate a disruption in endothelial communication as a contributor to overall impaired vascular reactivity. Additionally, myoendothelial junction presence and function can be evaluated through movement of dye between specific proteins found within gap junctions, called connexins (70). Negative staining for gap junction-specific connexin isoform phosphorylation and a lack of dyed amide transfer between ECs and SMCs at day seven would also support disrupted endothelial communication as a contributor to vascular reactivity impairment.

Pre-Existing Collateral Arteriogenesis

Pre-existing collaterals (PECs) are present in the spinotrapezius muscle of the Balb/C strain approximately 50% of the time. While these PECs differ from arterialized collateral capillaries in their anatomical position and remodeling methods, they can provide insight into the physiology of ACCs, without the influence of genetic differences

between Balb/C mice and other strains with pre-existing collaterals (26, 27). Smooth muscle cells (SMCs) surround PECs prior to arterial occlusion, allowing for arteriogenesis, while the collateral capillaries lack SMCs until they arterialize. The presence of existing smooth muscle on the PECS allows the cells to proliferate and reorganize in response to increased shear stress, whereas ACC smooth muscle must be recruited, potentially varying the methods and capacities to vasodilate between the vessel types (39). Both ACCs and PECs undergo remodeling, but the key differences in size and recruitment versus proliferation support the potential variation in reactivity between the vessel types at day seven.

Arteriogenesis involves vessel enlargement in response to changes in shear stress, for example, when blood supply is redirected as a result of an occlusion (26). When pre-existing collateral vessels (PECs) are present, blood flow will follow the path of least resistance and travel through the existing collateral network, around the occlusion (**Figure 13**). The initial drop in pressure and redirection of blood flow post-ligation will increase shear stress and cause the release of vasoactive paracrine factors. These factors include growth factors and vasodilators, such as endothelium-derived hyperpolarizing factor (EDHF) and prostaglandins, all of which contribute to the function and structure of vascular smooth muscle cells (22, 36). In the chronic setting of increased shear, as occurs following an occlusion, smooth muscle cells proliferate and differentiate, allowing for outward remodeling (22, 63). Shear stresses also increase in the capillaries that connect arterial branches and similarly activate the endothelium, but the capillaries also require the recruitment of smooth muscle cells to function as resistance vessels. In either case, the remodeling process requires the division and phenotypic transition of ECs and SMCs,

likely impairing their ability to effectively relax and contract when they are not expressing their primary phenotypes.

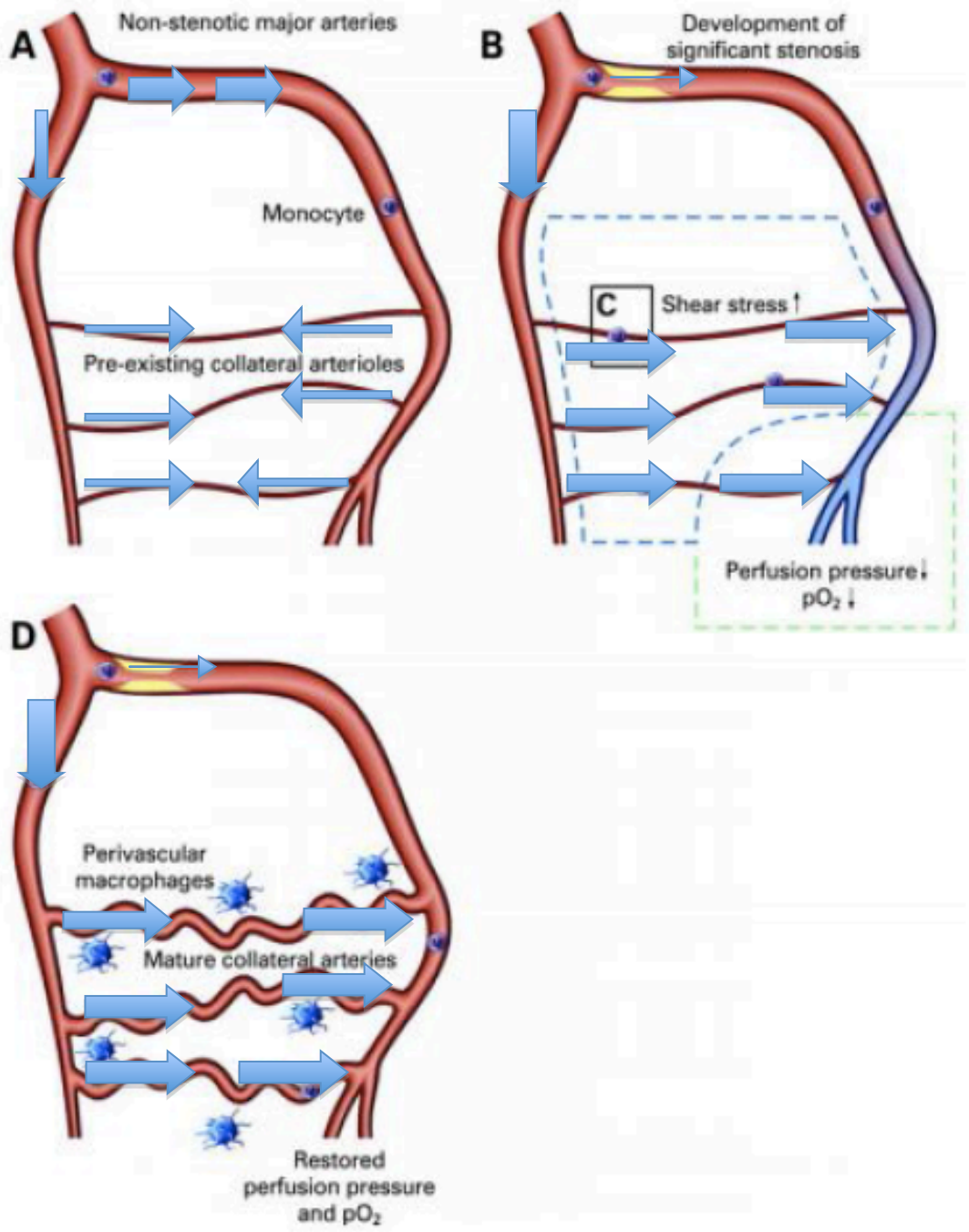

Figure 13. Blood flow and shear stress shift inducing collateral vessel development. Figure adapted from Schirmer (63).

While trending towards impaired vasodilation, the pre-existing collaterals did not dilate less than the sham arterioles, except in response to SNP. This lack of difference can be explained by insensitivity to nitric oxide and/or by an inequality in baseline diameters, as there were few to no contralateral arterioles that matched the large baseline diameter of the PECs. If the PECs developed a tolerance to nitrates following nitrate-mediated changes as the PEC develops in response to increased shear stress, NO-based vasodilation may be impaired and explain the lack of response (3). This could be supported if pharmacological evaluation with nitrates, like nitroglycerin and isosorbide-dinitrate, reveals PEC tolerance and insensitivity to NO. Additionally, reactivity of vessels depends on vessel size, in that the larger-diameter and higher-order vessels are capable of attaining larger percent changes in vasodilation (8). Because the baseline diameters of the sham arterioles are smaller on average than the PECs, the vessels are likely not equivalent in vasodilatory capability, and the PECs appear more reactive when compared against the smaller percent increase in sham diameter than they would if they were compared against a larger percent increase in equivalently-sized sham diameters.

PEC Vasodilation Via Electrical Signaling from EC to SMC

Because vasodilation signaling is typically linear from the endothelium to the smooth muscle via NO, prostaglandin, and/or endothelium-derived hyperpolarizing factor (EDHF) pathways, it was unexpected to find impairment in response to an endothelial-independent agent (SNP), but not in upstream endothelial-dependent agents (ACh), as occurred in the PECs at day seven. One explanation for the ability of the ACh-induced dilation to propagate despite an apparent impairment in NO signaling is that alternative pathways exist that bypass the dysfunction. One alternative that was not addressed in

isolation by the reagents is the direct evaluation of electrical coupling between endothelial cells (ECs) and smooth muscle cells (SMCs), which is capable of inducing dilation without the effects of NO, prostaglandins, and EDHFs in resistance microvessels (18). Electrical coupling can be measured directly with highly sensitive and precise receiving electrodes to detect any changes in membrane potential in the ECs or SMCs in accordance with reagent application. A high level of activity and membrane potential change following vasodilator application along with vasodilation inhibitors of the cellular pathways, would indicate electrical communication outside of the cellular cascades, and support electrical coupling in PECs as an alternative pathway.

Summary

Seven days following arterial occlusion, arterialized collateral capillaries (ACCs) and pre-existing collaterals (PECs) are capable of dilating and constricting in response to both endothelial-dependent dilators, endothelial-independent dilators, and an endothelial-independent constrictor; however, this capacity is largely impaired with respect to the reactivity of contralateral equivalent arterioles on the unoperated sides. This impairment is likely smooth muscle-based and may be explained by a variation in smooth muscle cell phenotype, extracellular matrix (ECM) remodeling, lack of innervation, and endothelial cell communication disruption within the ACCs, and by varied smooth muscle phenotype, ECM remodeling, and arteriogenic remodeling within the PECs. The ability for PECs to dilate normally in response to endothelial-dependent agents but not in response to endothelial-independent agents is likely because the former initiates alternative pathways such as electrical coupling between the endothelium and smooth muscle. This electrical coupling can successfully propagate a vasodilation signal

independent of the more common cellular pathways such as NO, prostaglandin, and EDHF. These findings that the PECs may employ alternative pathways to dilate and that both ACCs and PECs are capable of dilation following ischemic events improve our understanding of collateral development. This knowledge can advance treatments of increasingly prevalent cardiovascular diseases by identifying and utilizing some of these alternatives as avenues to regain vessel reactivity.

Chapter III. ACC AND PEC REACTIVITY AT DAY-21

INTRODUCTION

Summary

At 21 days post-ligation, arterialized collateral capillaries (ACCs) and pre-existing collaterals regain functionality and demonstrate vasodilation capabilities comparable to contralateral arterioles following muscle contraction (13). The 'mature' vessels have had more time to develop than those at seven days following ligation, and have likely overcome the limitations of successful dilation with fully differentiated and arranged smooth muscle cells, integrated ECM, and/or appropriate innervation. Because dysfunction was so prevalent at day seven, however, it is possible that these recently adapted collaterals are more or less dependent on certain pathways or utilize alternative pathways to dilate successfully (23, 37). To investigate ACC and pre-existing collateral (PEC) dependence on the prostaglandin and nitric oxide synthase (NOS) pathways for vasodilation, we applied vasodilation inhibitors indomethacin and L-NAME to the vessels 21 days post occlusion during electrode-induced muscle contraction. Indomethacin significantly reduces vasodilation in healthy terminal arterioles, L-NAME has no effect (61), and we predict the vessels will be mature at day 21. Thus, we hypothesize that ACCs and PECs will be at least partially dependent on the prostaglandin and not the NOS vasodilation pathways, such that dilation is impaired by indomethacin and unimpaired by L-NAME application to approximately the same degree as in the respective control vessels.

Prostaglandin and NOS-Based Pathways

Endothelial cells are involved in successful vessel dilation through purinergic receptor stimulation and an increase of local calcium ions (17). Thus, investigating

dysfunction in the prevalent endothelial-derived relaxing factor (EDRF) production pathways within the endothelium may reveal contributors to functional vasodilation impairment, as demonstrated at day seven. EDRFs primarily describe three vasodilatory signals that originate from the endothelium and include nitric oxide (NO), prostaglandin I_2 (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF) (24). EDHF is more critical in smaller resistance vessels such as arterialized capillaries, while in larger pre-existing collaterals, NO production may be more prevalent; however, PGI_2 mediates endothelial-dependent vasodilation in both the smaller and larger arterial vessels (22, 73). EDHF hyperpolarizes the membrane by activating potassium channels on the smooth muscle to relax the cells, and PGI_2 activates adenylyl cyclase (AC) by binding to prostaglandin I_2 (IP) receptors and activating G-protein coupled receptors specific to AC. Adenylyl cyclase increases cAMP levels and activates PKA to, ultimately, decrease intracellular calcium levels and relax the vascular smooth muscle in vasodilation. The cyclooxygenase (COX) pathway is followed by members of the eicosanoid family, integrating oxygen as a major cosubstrate to induce vasodilation via prostacyclin (PGI_2) synthesis (20). PGI_2 then potentiates the signal by activating AC and leading to SMC relaxation. Alternatively, NOS converts L-arginine into nitric oxide (NO), which relaxes the smooth muscle by directly activating soluble guanylyl cyclase (30, 38, 71). Guanylyl cyclase produces cGMP, which activates PKG to inhibit myosin light chain kinase and reduce intracellular calcium (5, 40, 75).

We hypothesized that the ACCs and PECs would be at least partially dependent on the prostaglandin pathway and not dependent on the NOS pathway at 21 days post-occlusion. Dependence would manifest as a significantly decreased functional

vasodilation following application of prostaglandin and NOS dependent pathway inhibitors. An equal or varied dependence on these pathways as compared to arterioles on the unoperated sides will provide more insight on the development of the collaterals vessels in response to an ischemic event (37). With an improved understanding of what dilation pathways are impaired and what pathways function well in the early stages of ACC and PEC development, we may be able to identify factors to expedite and support the development and maturation of collaterals. 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 (58). By facilitating efficient development of these collateral networks, we may improve the prognosis of patients with less advantageous vasculature to cope with diseases like PAOD.

METHODS

Animal Care and Housing

Male Balb/C mice were cared for and utilized, as described in Chapter 2, under the guidelines specified and protocols approved by the Cal Poly State University SLO Institutional Animal Care and Use Committee.

Spinotrapezius Lateral Feed Artery Ligation

To stimulate the arterialization of capillaries, the lateral spinotrapezius feed artery was ligated following the same procedures as described in Chapter 2.

Vasodilation Inhibition Testing

To ensure the efficacy of the vasodilation inhibitors, dilators specific to their pathways were applied before and after inhibitor application. The two vasodilation pathways addressed were prostaglandin and nitric oxide synthase (NOS). Arachidonic acid (AA) metabolizes into hydroxyeicosatetraenoic acids (HETE), which, ultimately, increase voltage gated calcium channel sensitivity to reduce intracellular calcium levels and relax smooth muscle cells. This process utilizes the prostaglandin pathway, which can be inhibited by indomethacin downstream of AA activation to analyze the prostaglandin pathway more exclusively (56, 61). Indomethacin is a non-steroidal anti-inflammatory drug (NSAID), which inhibits cyclooxygenase (COX) and prostaglandin synthesis (24, 71).

Regarding nitric oxide (NO) dependent pathways, acetlycholine dilates the endothelium upstream of where L-NAME inhibits NO formation. N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) binds to nitric oxide synthase (NOS) and inhibits nitric oxide (NO) production, thereby inhibiting endothelial-dependent vasodilation (36).

When used alone, L-NAME constricts the local blood vessels; however, when used together, sodium nitroprusside and L-NAME can prevent endothelial-dependent vasodilation while maintaining a pre-determined baseline vessel diameter.

Appropriate concentrations of indomethacin and L-NAME were optimized to sufficiently inhibit vasodilation in the prostaglandin and NOS pathways by applying increasing amounts of either vasodilation inhibitor until the effects of the respective dilators were minimized. The superfusion protocol from Chapter 2 was used with $2x10^{-4}$ M arachidonic acid and 10^{-5} M acetylcholine to stimulate the prostaglandin and NO based dilations, inhibited respectively by $2x10^{-4}$ M indomethacin and $2x10^{-5}$ M L-NAME. Testing was also performed to confirm consistent functional vasodilation in response to repetitive electrode stimuli throughout the duration of the experiment (Appendix A). Vessel diameter was continuously monitored along with post-wait dilation capability to determine stabilization and confirm 30 minutes as a sufficient waiting period.

Vascular Reactivity with Intravital Microscopy - Functional

Twenty-one days following each ligation surgery, the spinotrapezius muscle was re-exposed. The same superfusion protocol was followed, as described in Chapter 2, with an added element of functional stimulation and each of the following inhibitory agents were independently administered to the area via superfusion: $2x10^{-4}$ M indomethacin and $2x10^{-5}$ M L-NAME. SNP was added as necessary to maintain baseline diameters with L-NAME exposure in 10^{-7} M aliquots. Muscle-stimulating electrodes were placed on the caudal end of the spinotrapezius muscle, where a test contraction was detected at 1 Hz, 2 mA, and 200 μs duration to confirm placement onto the stimulus site. Once the stimulus site was located, as determined by consistent muscle twitching, stimulation ceased, the

electrodes were left in place, and the superfusion equipment was positioned to allow physiological salt saline (PSS) to flow over the muscle (**Figure 14**).

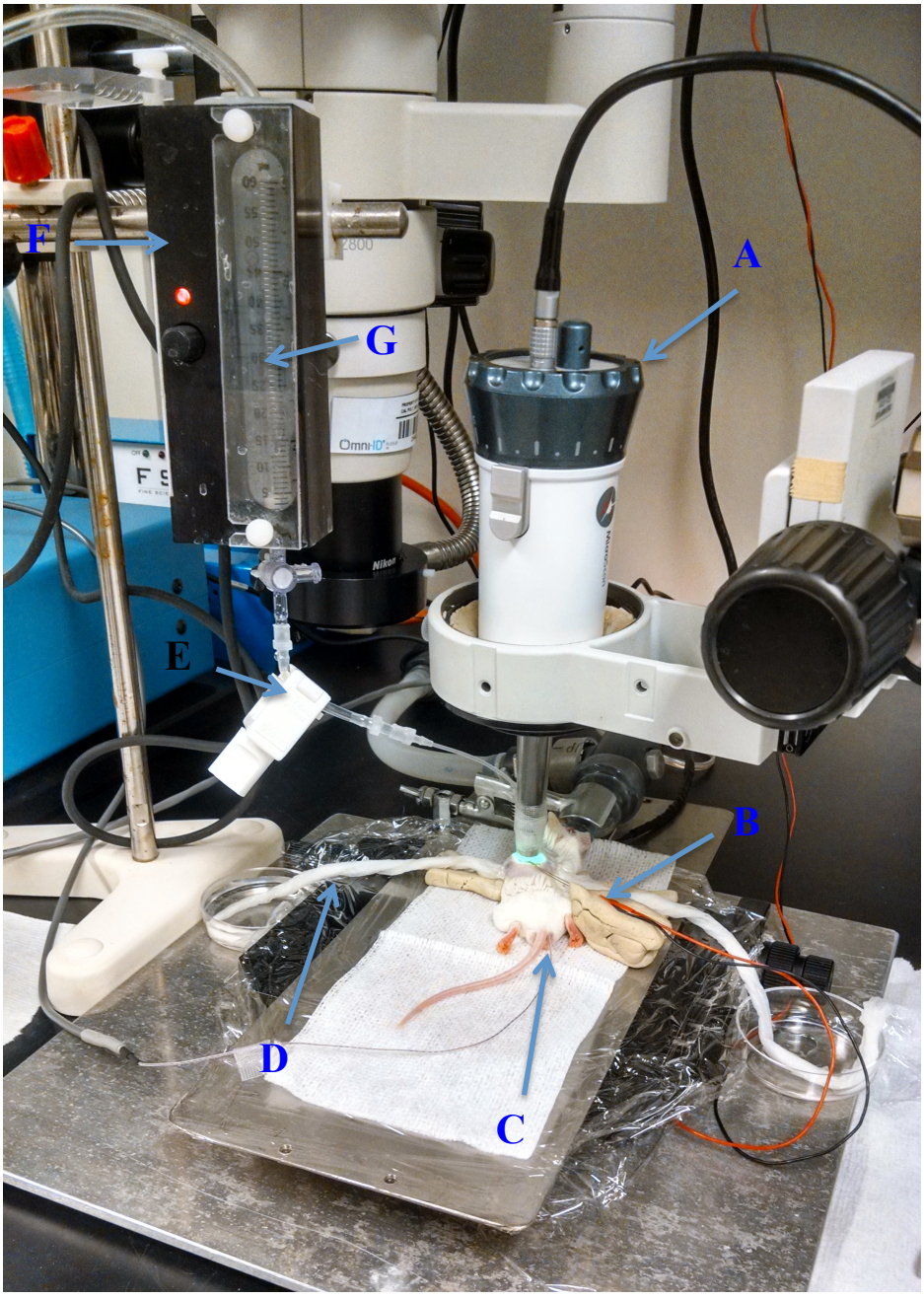

Figure 14. Superfusion preparation with intravital microscope and electrodes. Electrodes placed at caudal end of spinotrapezius to stimulate contractions and exercise-induced vasodilation with simultaneous superfusion delivery of reagents. **A)** Microscope, **B)** stimulating electrodes, **C)** rectal temperature probe, **D)** wick to remove excess fluid, **E)** flow regulator and tubing, **F)** syringe heater, **G)** 60 mL syringe filled with physiological salt solution (PSS).

With the equipment in position, electrode-induced muscle contraction was tested again. The vessel(s) of interest were imaged after a 30-minute equilibration period to record baseline diameters, and the muscle was stimulated for 90 seconds at 8 Hz, 2 mA, and 200 μs duration. Vessels were imaged again immediately following stimulation to record a control dilation response.

During the following 30-minute wait period between stimuli, $2x10^{-5}$ M L-NAME, $2x10^{-4}$ M indomethacin, or both were applied via superfusion for a minimum of 15 minutes. L-NAME and indomethacin were applied in random order, followed by both applied simultaneously. Vessels were imaged pre and post-stimulation for each combination of vasodilation inhibitors, and the functional stimulation with superfusion was repeated on the contralateral limb. Arterialized capillaries or pre-existing collaterals were analyzed on the ligated side, and equivalently-sized arterioles were analyzed on the sham side. Both the ligated and sham spinotrapezius muscles were also removed, fixed overnight in 4% paraformaldehyde, and stored in PBS.

Imaging and Statistical Analysis

The images/videos were analyzed using AVA software to compare diameters before and after reagent application in the sham and arterialized collateral capillaries or pre-existing collaterals. Differences in resting and dilated/constricted diameters and percent changes were evaluated by homoscedastic t-tests and one-way ANOVA. Data are presented as means ± standard error.

RESULTS

To test the hypothesis that the mature arterialized collateral capillaries (ACCs) and pre-existing collaterals (PECs) are at least partially dependent on the prostaglandin and not on the nitric oxide synthase based pathways within the endothelium, we analyzed reactivity in the more developed vessels at 21 days following ligation. The spinotrapezius muscles were functionally stimulated with electrodes to prompt exercise-induced vasodilation while the vessels were simultaneously exposed to vasodilation inhibitors, indomethacin and L-NAME. These reagents are specific inhibitors of the prostaglandin and nitric oxide synthase pathways so that a significant decrease in ACC or PEC reactivity, as compared to the control vessels, would indicate a greater dependence on the inhibited pathway for proper dilation.

L-NAME and indomethacin successfully prevented acetylcholine and arachidonic acid-induced vasodilation, respectively, in healthy arterioles (**Figure 15**).

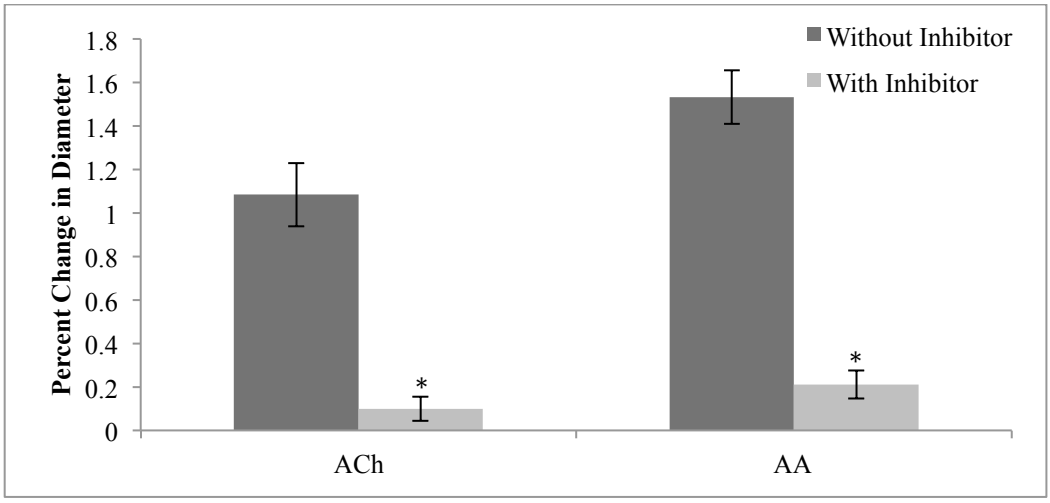

Figure 15. Inhibition of ACh and AA induced vasodilation. Percent changes of vessel diameter in response to dilators ACh (n=6) and AA (n=4) with and without inhibitors L-NAME and indomethacin, respectively * indicates $p < .05$ using a homoscedastic t-test.

L-NAME reduced the percent increase in diameter in response to acetylcholine (ACh) from 108.5 ± 14.5 % to 10 ± 5.5 %. Indomethacin reduced the percent increase in diameter in response to arachidonic acid (AA) from 153.5 ± 12.5 % to 21 ± 6.5 %.

Vasodilation was not inhibited in the arterialized collateral capillaries (ACCs) nor in the contralateral arterioles in response to all combinations of L-NAME and indomethacin with functional stimulation (**Figure 16**). ACC diameters increased from 9 ± 0.5 μm to 19 ± 1.5 μm in response to functional stimulation, from 8 ± 0.5 μm to 15.5 ± 2.5 μm with L-NAME applied, from 8.5 ± 1 μm to 19.5 ± 2 μm with indomethacin applied, and from 9 ± 0.5 μm to 17.5 ± 2 μm with both L-NAME and indomethacin applied. Sham arteriole diameters increased from 8.5 ± 0.5 μm to 19.5 ± 1.5 μm in response to functional stimulation, from 8.5 ± 0.5 μm to 16.5 ± 1.5 μm with L-NAME applied, from 9 ± 0.5 μm to 21.5 ± 2 μm with indomethacin applied, and from 8.5 ± 0.5 μm to 19 ± 1.5 μm with both L-NAME and indomethacin applied.

The percent increases in diameter were comparable between the sham and ligated sides and also between every reagent. Diameters in the ACCs increased 124 ± 27 % with no reagent applied, 90 ± 25 % with L-NAME applied, 119.5 ± 28 % with indomethacin applied, and 94.5 ± 14.5 % with both applied. Similarly, diameters in the sham arterioles increased 105 ± 18 % with no reagent applied, 81.5 ± 19 % with L-NAME applied, 119.5 ± 20 % with indomethacin applied, and 111 ± 15.5 % with both applied. This lack of response in both the sham and ligated sides is interesting considering the strong inhibitory effects of L-NAME and indomethacin on acetylcholine and arachidonic acid-induced vasodilation in healthy arterioles.

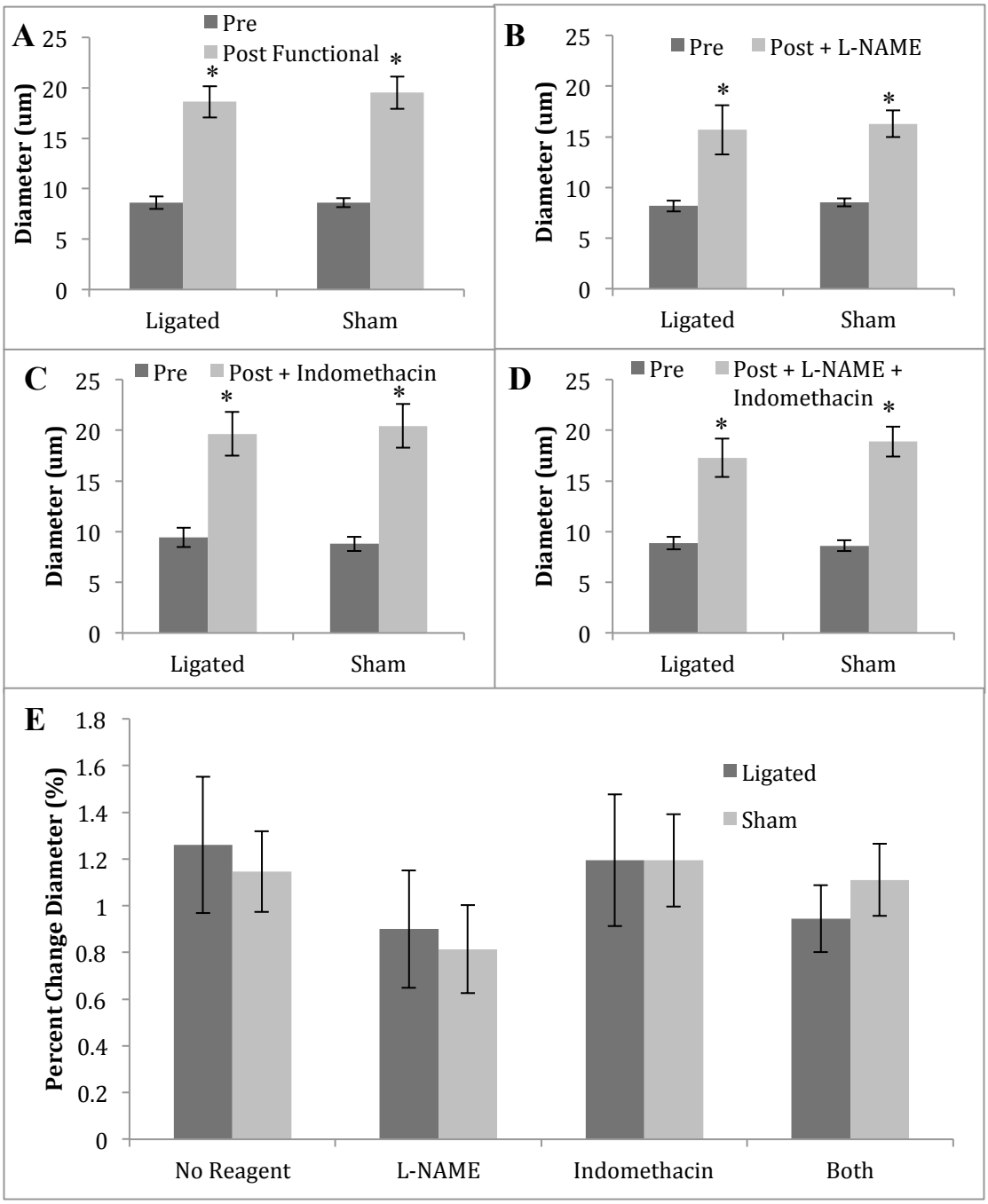

Figure 16. Arterialized capillary reactivity at day 21. Diameters (μm) pre and post exposure to functional stimuli with no reagent **(A)**, $2x10^{-5}$ L-NAME (n=7) **(B)**, $2x10^{-4}$ indomethacin (n=7) **(C)**, or both $2x10^{-5}$ L-NAME and $2x10^{-4}$ indomethacin **(D)** added. **E)** Percent changes of vessel diameter in response to functional stimuli with and without reagents**;** * indicates $p < .05$ using a homoscedastic t-test.

To analyze the pre-existing collaterals (PECs) as a basis for comparing ACC reactivity to a more studied vessel type, the functional superfusion protocol was applied with the two vasodilation inhibitors, L-NAME and indomethacin, at 21 days post-ligation (**Figure 17**). At the later time-point, the PEC midzones trended towards lower percent increases in diameter with no reagent, L-NAME, and both L-NAME and indomethacin applied. PEC midzone diameters increased from 24.5 ± 6 μm to 41 ± 5 μm in response to functional stimulation, from 20.5 ± 4 μm to 38.5 ± 7 μm with L-NAME applied, from 33.5 ± 8.5 μm to 43.5 ± 5.5 μm with indomethacin applied, and from 23 ± 4.5 μm to 39.5 ± 7 μm with both L-NAME and indomethacin applied. Sham arteriole diameters increased from 15 ± 2.5 μm to 37.5 ± 6 μm in response to functional stimulation, from 18 ± 5.5 μm to 46 ± 9.5 μm with L-NAME applied, from 19 ± 4 μm to 46.5 ± 7 μm with indomethacin applied, and from 18 ± 4.5 μm to 43 ± 10.5 μm with both L-NAME and indomethacin applied.

Indomethacin application led to the only significant decrease in percent change at the PEC midzone as compared to the arterioles on the sham side. Diameters in the PECs increased 81.5 ± 35 % with no reagent applied, 80 ± 18 % with L-NAME applied, 23 ± 21.5 % with indomethacin applied, and 107 ± 48 % with both applied. Alternatively, diameters in the sham arterioles increased 162 ± 25 % with no reagent applied, 158.5 ± 34 % with L-NAME applied, 151.5 ± 15.5 % with indomethacin applied, and 148 ± 7 % with both applied.

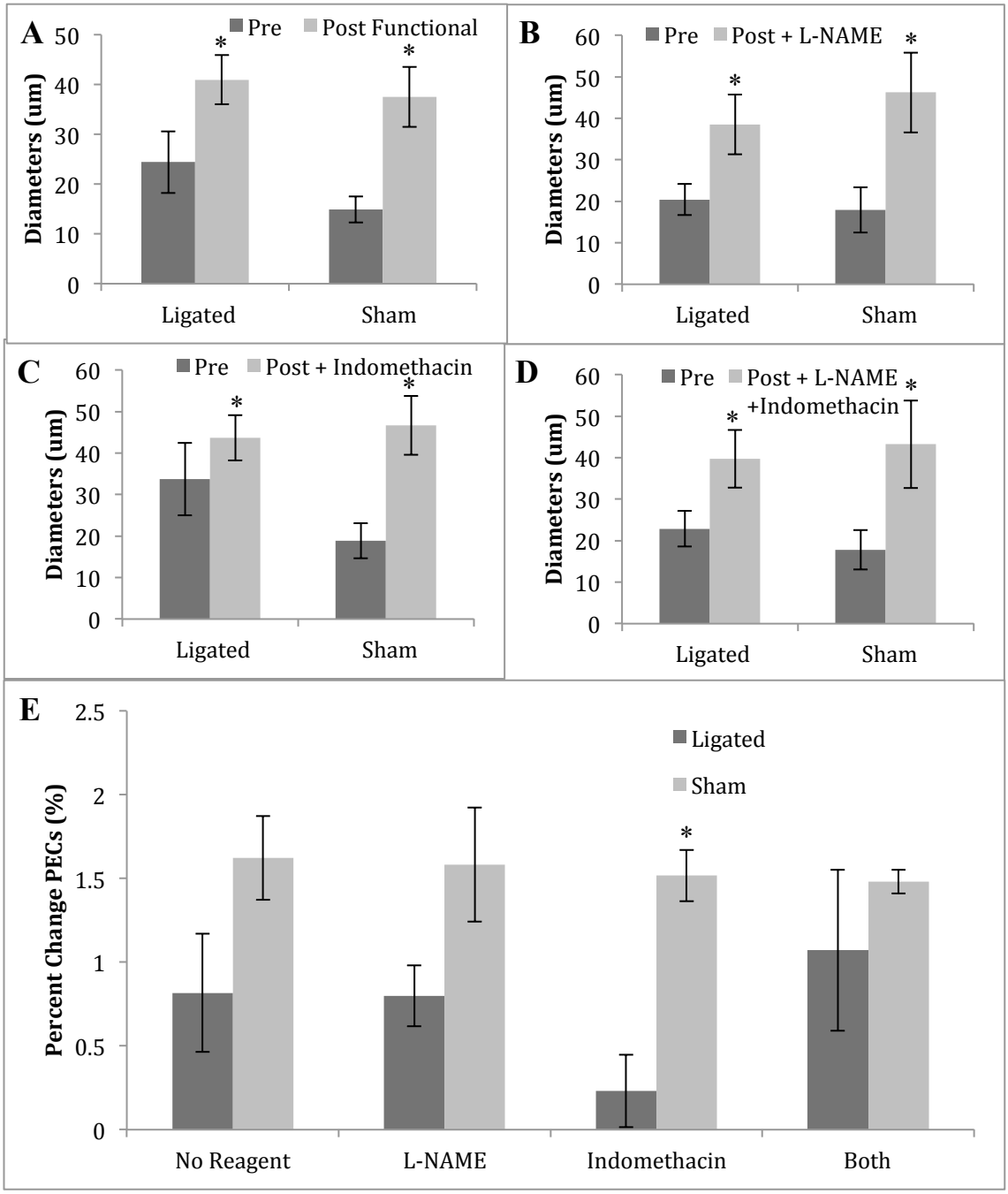

Figure 17. Pre-existing collateral reactivity at day 21. Diameters (μm) pre and post exposure to functional stimuli with no reagent **(A)**, $2x10^{-5}$ L-NAME (n=7) **(B)**, $2x10^{-4}$ indomethacin (n=7) **(C)**, or both $2x10^{-5}$ L-NAME and $2x10^{-4}$ indomethacin **(D)** applied. **E)** Percent changes of vessel diameter in response to functional stimuli with and without reagents**;** * indicates $p < .05$ using a homoscedastic t-test.

The three main portions of each pre-existing collateral were also measured and analyzed to observe any variation within the vessels themselves (**Figure 18**). Location of diameter measurements could be critical to accuracy if the reactivity or baselines are different between the regions of the PECs. All three regions – reentry, midzone, and stem – significantly dilated in response to functional stimulation, as seen in **Table I**.

Table I. Responses at Various Locations along PEC at Day 21

		Reentry (μm)	Midzone (μm)	Stem (μm)
No Reagent	Pre	27.4 ± 4	24.5 ± 6	21.5 ± 4.5
	Post	57 ± 7	41 ± 5	38.5 ± 5
L-NAME	Pre	26.5 ± 4	20.5 ± 4	17.5 ± 3.5
	Post	55.5 ± 9	38.5 ± 7	32.5 ± 7.5
Indomethacin	Pre	38.5 ± 10.5	33.5 ± 8.5	25 ± 4
	Post	54 ± 9.5	43.5 ± 5.5	42 ± 4.5
Both L-NAME + Indomethacin	Pre	29 ± 4	23 ± 4.5	19.5 ± 3
	Post	52.5 ± 10.5	39.5 ± 7	36.5 ± 7.5

Only the reentry and stem regions dilated in response to functional stimulation with L-NAME applied, only the stem region dilated in response to functional stimulation with indomethacin applied, and only the reentry and stem regions dilated in response to functional stimulation with both L-NAME and indomethacin applied. There was a trend in decreasing maximum diameter reached from the reentry to the stem of the preexisting collateral, though each portion of the PEC had the capacity to significantly dilate. Indomethacin trended towards reducing the percent increase in diameter throughout the length of the PECs as compared to the other conditions of functional vasodilation.

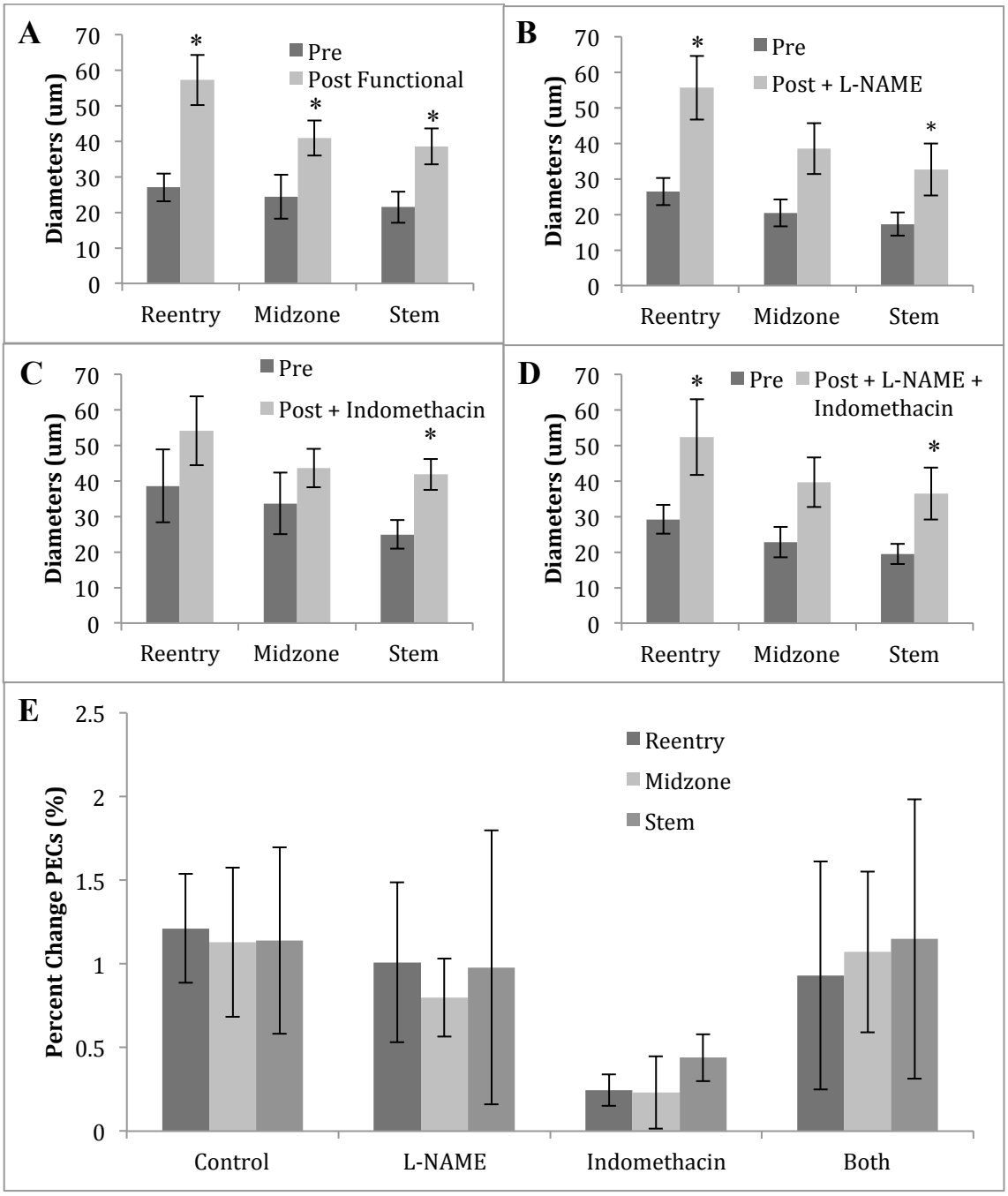

Figure 18. Pre-existing collateral component reactivity. Diameters (μm) pre and post exposure to functional stimuli at the stem, midzone, and reentry regions with no reagent **(A)**, $2x10^{-5}$ L-NAME (n=7) **(B)**, $2x10^{-4}$ indomethacin (n=7) **(C)**, or both $2x10^{-5}$ L-NAME and $2x10^{-4}$ indomethacin **(D)** applied. **E)** Percent changes of vessel diameter in response to functional stimuli with and without reagents**;** * indicates $p < .05$ from rest using a homoscedastic t-test.

ACCs and PECs both have the potential to remodel; however, ACCs must first recruit the smooth muscle cells before they can proliferate, whereas PECs can initiate this process immediately in response to increased shear stress. To further compare ACCs and PECs in their development and remodeling processes, the progression of baseline diameters was compared between arterialized collateral capillaries and pre-existing collaterals at zero days (unligated), seven days, and 21 days post-ligation (**Figure 19**).

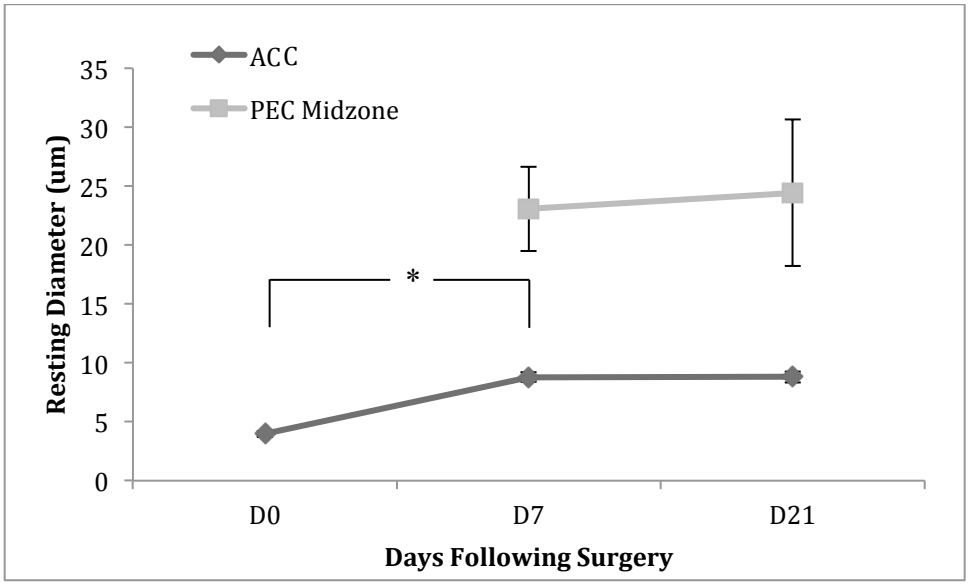

Figure 19. Baseline diameter progression of ACCs and PECs. Progression of arterialized collateral capillary (ACC) baseline diameters from day 0 (n=7), day 7 (n=16), and day 21 (n=10), along side pre-existing collateral (PEC) baseline diameters at day 0 (n=1), day 7 (n=10), and day 21 (n=5); * indicates $p < 0.001$.

Baseline diameters for the PECs remained relatively constant from day seven to day 21 (23 ± 3.5 μm to 24.5 ± 6 μm); however, arterialized capillary diameters significantly increased from pre to seven days post-ligation (4 ± 0.5 μm to 9 ± 0.5 μm). PEC baseline diameters were not collected at day zero, so parallels cannot be drawn at that time point.

DISCUSSION

Arterialized collateral capillaries (ACCs) and pre-existing collaterals (PECs) provide alternative routes for blood flow in the event of an occlusion and can reperfuse the otherwise ischemic tissue (25, 79). Although significantly impaired at day seven, ACCs regained their ability to vasodilate in response to functional stimulation by 21 days post-ligation when compared to their equivalently-sized counterparts on the unligated limb (13, 26). PECs were impaired at day seven, but seem to continue the trend at day 21. The capacity to respond to this stimulus is crucial to maintain sufficient blood flow to areas when exercising, for example (29). It is possible that, because these vessels have developed from capillaries, they may utilize alternative vasodilation pathways than do normal arterioles (23, 37). We fail to reject the hypothesis that these seemingly mature collaterals depend on the same vasodilation pathways in comparison to arterioles on the contralateral side.

Mature Arterialized Collateral Capillary Responses

The ACCs and sham vessels responded equally to electrode-induced muscle contraction with and without various combinations of vasodilation inhibitors, with diameters increasing 124 ± 27% in ACCs and 105 ± 18% in sham arterioles with no reagents applied. The renewed ability to dilate suggests that ACCs are fully mature at day 21 and function with the same capacity as healthy arterioles; however, the lack of response to the inhibitors by either vessel type was unexpected. An explanation for the lack of response to the reagents is that the concentrations were insufficient to block the dilation pathways within the functional stimulation context. The doses were calculated to be strong enough to block potent, though specific, dilators arachidonic acid (AA) and

acetylcholine (ACh) for indomethacin and L-NAME, respectively, though, this might not be sufficient to overcome the other pathways triggered by the muscle contractions (20). In the future, increasing doses of inhibitors or inhibitors of alternative pathways could be used to investigate any increased efficacy in reducing functional vasodilation capabilities in healthy vessels. Once the effective inhibitors are potent enough to have a significant impact on functional vasodilation in control arterioles, we can move forward with evaluating the developed collateral vessels.

The vessels may also use alternative pathways outside of the prostanoid and NOS pathways to dilate successfully (1). Though it is still possible that arterialized collateral capillaries utilize alternative vasodilation pathways that were not evaluated in this study, our hypothesis that arterialized collateral capillaries would be partially dependent on the prostaglandin and nitric oxide synthase pathways is supported.

Mature Pre-Existing Collateral Responses

In efforts to correlate the reactivity and development of ACCs with that of the more studied pre-existing collaterals (PECs), PECs were analyzed whenever present in every experiment (63). PECs trended towards impaired vasodilation at day seven in response to endothelial-independent dilators, but had a normal response to endothelial-dependent dilators. The trend of impairment continues in response to functional stimulation and to the prostaglandin and nitric oxide synthase (NOS) based pathways at 21 days following ligation. This continued trend indicates that, though the PECs may be mature, their capacity to dilate may be inherently lower than native arterioles. Alternatively, the impairment is not significant, and the unequal percent changes could be explained by PEC baseline diameters being larger than resting. The inaccurately high

resting diameter would reduce the percent change to maximal dilation and indicate impairment that may not exist. A trend in impaired dilation may be due to a variation in smooth muscle phenotype, which has varied compositions of actin and myosin that are not as effective at relaxing and contracting (57). A variation in phenotype could be identified through histological analysis of resected samples of the PECs. Staining for specific markers, such as through immunostaining with smooth muscle α-actin, within the PECs and sham arterioles may reveal differences between the two vessel types (26).

The inhibited dilation in response to indomethacin indicates dependency on the prostaglandin pathway in all three PEC regions; however, this may be explained by an offset baseline. PEC diameters increased from 24.4 ± 6.2 μm without any reagents to 33.7 ± 8.7 μm with indomethacin exposure. Thus, the decreased percent increase in diameter may be the result of increased sensitivity to cyclooxygenase (COX) derivatives, as COX and nitric oxide synthase (NOS) have been linked in expression and activity (71). If COX is inhibited by indomethacin, it has the potential to interfere with those interactions and increase NOS activity, thereby, relaxing the smooth muscle cells, elevating baseline diameters, and reducing the percent increase required to reach a maximum diameter. The PEC diameter responses were also highly variable, with standard errors as high as 48%; thus, a more accurate evaluation of PEC reactivity may be achieved by lowering response variability through measuring PEC regions with more similar baseline diameters. Starting with the same diameter implies that the vessels will have the same capacity for dilation, allowing for more consistent responses to the same amount of reagent and/or electrode stimulation.

Other ways to reduce variability in vascular reactivity include measuring at the same area along the vessel consistently with all reagents on each side, to be gentle when exposing and probing the muscle tissue, and to maintain clean equipment and pure reagents. Measuring at alternative locations is acceptable in consecutive experiments as the relative reactivity should be consistent throughout the reagents; however, measuring at different locations within the same data set may result in high diameter variability as each zone or vessel level may have a greater or weaker capacity to respond (8). Aggressive probing or damage during exposure and analysis leads to a response from the tissue, whether it be vasodilation or constriction. This external stimulation could confuse and diversify the results with either exaggerated or inhibited reactivity. Occasionally, impurities within the superfusion tubing line or in the PSS would lead to vascular responses independent of the reagents or electrode stimulation. Thus, maintenance of clean tubing and fresh solutions may eliminate responses to contaminants as a variable and allow for more consistent diameter measurements.

In both ACCs and PECs, baseline diameters are generally unchanging from seven to 21 days post-ligation; though, diameters increase from pre-ligation to seven days post-ligation in the ACCs. The initial outward remodeling resulting in diameter increases seems to occur in the first seven days, followed by a plateau of relatively stable diameters. If the diameter changes are associated with vessel maturity, the restored capability of ACCs to dilate at day 21 in response to electrode-induced muscle contraction would also be expected in the PECs (8). The impaired capacity for PECs to dilate may correlate with an impaired ability to remodel, as supported by collateral vessels having impaired function in the Balb/C strain, potentially due to a lower

expression of VEGF-A (11, 52). VEGF-A is a critical cytokine in vascular development and is linked with TNF-α levels, which are specifically important to remodeling in collateral vessels (11, 74, 76). When present, VEGF-A may prompt arteriogenesis and enhance collateral blood flow by stimulating monocyte migration (74). VEGF-A levels could be identified through tissue processing, RNA extraction, PCR, and electrophoresis. If the PECs had a relatively lower expression as compared to arterioles of equivalent size and ACCs, this explanation would be supported. PECs in the Balb/C are very distinct post-ligation, as they tend to connect the cranial and caudal feeds and follow the vein pairs closely, but with a very large and tortuous form. Alternatively, ACCs tend to branch in an angled or perpendicular fashion and connect the same feeds with smaller, less tortuous, and more numerous vessels. The anatomical differences further suggest a variation in growth factor prevalence, as their balance is crucial in the development of the vessels (76).

The trend towards increased maximum diameters from the reentry, through the midzone, and to the stem may be indicative of increased remodeling along the vessel from the stem to the reentry. Shear flow not only increases throughout the PECs, but the direction of flow changes for the mid and reentry zones, as blood flows upstream towards the occlusion site. Shifts in shear stress encourage remodeling, so that the re-entry, which experiences the largest directional shift in flow, may have also remodeled the most (8). This increased remodeling, however, is not supported by the similar reactivity within each region at the mature time-point of 21 days post-ligation. It may be the case that there is no difference in reactivity along the length of the PECs, as the changes in diameter are

not statistically different. This would indicate that measurements could be taken at any point on the PEC for analyzing vessel reactivity.

Chapter IV. DISCUSSION

The arterialization of collateral capillaries in animals lacking pre-existing collateral arterioles can re-establish blood flow to an ischemic vascular tree (13, 25); however, to match blood flow with tissue demand in the reperfused areas, collaterals and/or arterialized collateral capillaries (ACCs) need to vasodilate. Although the ACCs acquire smooth muscle cells within seven days following the spinotrapezius lateral feed artery ligation, functional vasodilation is absent at this time point (13, 21, 25). We observed impairment in vessel reactivity at day seven; however, the arterialized capillaries maintained a capacity to dilate and constrict in response to the vasoactive agents. This discrepancy can be explained by an absence or impairment of endothelial communication, as capillaries are not normally a part of the conduction circuit. The direct application of reagents bypasses this communication and relaxes or contracts smooth muscle on-site.

The impairment that does exist in ACCs at day seven can be explained by vessel immaturity through variation in smooth muscle phenotype, ECM remodeling, and/or a lack of innervation; however, by day 21, the ACCs regain their ability to dilate and can be considered mature. 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, so that any smooth muscle is inherently new to the area or new altogether and may require an adjustment period. The specific time-point between seven and 21 days post-occlusion at which the vessels gain these functions is unknown.

When a pre-existing collateral (PEC) existed in the vasculature, blood flow presumably redirected through it immediately post-ligation, and robust arterialized

capillaries were consequently absent. With blood flow traveling the path of least resistance, it would exert increased shear stresses on the PECs rather than the capillaries, minimizing the stimulus for them to arterialize. PECs maintained a trend towards an inability to dilate or constrict effectively at day seven and day 21; however, only the NO and prostaglandin pathways were significantly impaired at day seven and 21, respectively. The impairment at day seven may be explained by ECM remodeling, as proposed for arterialized capillaries, and arteriogenesis, where the smooth muscle cells are in an adaptive phase and limited in their ability to dilate and constrict. On the other hand, endothelial-dependent dilation and constriction were normal at day seven, possibly because the vessels were able to utilize alternative pathways, such as electrical coupling, to dilate. If the PECs can dilate with these alternative pathways, this would indicate that the prostaglandin and hyperpolarization pathways, as evaluated through reagent application, also contribute to the dysfunction. The trend towards continued impairment at day 21 may be due to the nature of Balb/C mice in lacking robust collaterals with high levels of VEGF-A, and the significant decrease in functional response with indomethacin exposure is potentially instigated by interactions between COX derivatives and NOS elevating baseline diameters.

Limitations and Future Work

Because vasculature is less reactive when co-morbidities are present, our model of cardiovascular disease that utilizes young and healthy mice is limited (35, 59, 78). The arterialized collateral capillaries that formed in this study are likely more robust and functional than they would be in, for example, a mouse with diabetes (74). A more clinically accurate and relevant representation of vessel reactivity and development

would be in a diseased model of diabetes or hypertension, as these are prevalent byproducts and causes of cardiovascular disease in the world (28, 66). While no perfect model exists, hypertensive and diabetic mice can be developed through diet adjustment and/or genotyping in house or through a vendor, so that they can closely mimic human symptoms (33, 59). For example, hypertension can be induced with intracerebralventricular (ICV) sodium infusion in telemetered Nedd4-2 knockout mice, and the db/db mouse strain carries a mutation that naturally results type II diabetes when placed on an energy-rich diet (33, 59). Understanding any varied mechanisms in the diseased setting would be advantageous for evaluating the efficacy of naturally remodeled vessels or of exogenous treatment to enhance arteriogenesis. Thus, the proposed next study involves analysis of vascular reactivity in a diseased model utilizing similar evaluation methods and time-points of smooth muscle and endothelial interrogation via functional stimulation and superfusion at day seven and 21.

Although follow-ups for human clinical trials for cardiovascular disease and collateral monitoring are often six months or more, the shorter lifespan and genetic manipulability of rodents allows for accelerated and relatively accurate models to see significant changes at time-points in the days and weeks range (59, 65, 77). Additionally, the collateral remodeling processes begin immediately following the occlusion in response to the changes in shear stress, such that changes in wall area and blood flow are present within the first few days (25, 52). The seven-day time-point, in particular, is used in this study because it is a convenient date for to follow-up regarding experimental logistics, and it is a common date for collateral analysis (25, 48, 52). After approximately three weeks, these changes level off, supporting 21-days as an appropriate time-point to

analyze developed collaterals. Future work to expand on this research could also entail investigating some of the other time-points not analyzed within the study to track arterialized collateral capillary development. For example, ACCs and PCEs at day 14 may be at their final luminal diameters, but the intracellular and cellular vessel wall constituents may not be optimally arranged. Histological examination of the arterialized capillaries and pre-existing collaterals could also determine their precise cellular constituents throughout the process. Actin and myosin isoforms could be stained within the smooth muscle, along with collagen fiber density, integrin, and matrix metalloproteinase (MMP) presence in the ECM. Observing vascular wall components of the vessels 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 vascular development process, we may be able to stimulate or control it in a clinical setting.

By understanding the mechanisms behind the early impairment in arterialized capillaries and the consistent impairment in pre-existing collaterals, we may be able to minimize the duration and extent of ischemia by effectively increasing perfusion to downstream tissues. On the clinical front, the success of collateralization could be evaluated through local application of vasodilators and vasoconstrictors similar to those used in this study via catheterization and angiograms (51, 65). The changes in collateral flow index and luminal diameter would indicate reactivity or lack thereof within the vessels. Manipulating the arterialization of capillaries may improve PAOD patient prognosis by more quickly and appropriately providing blood flow to ischemic tissues downstream of an occlusion.

Summary

Vascular reactivity in arterialized collateral capillaries (ACCs) is impaired in their developmental stages, likely due to smooth muscle cell immaturity described by phenotype variation and abnormal actin and myosin levels. By 21 days post-occlusion, the ACCs reached a capacity to dilate and constrict equal to unoperated arterioles of equivalent size. At both seven and 21 days, pre-existing collaterals maintained a trend of impaired reactivity, as supported by smooth muscle dysfunction, weak remodeling capabilities, and low VEGF-A expression in the Balb/C mouse strain (11). Despite this smooth muscle-based impairment trend, PECs may utilize electrical coupling or alternative pathways to dilate or constrict in response to endothelial-dependent agents. While early impairment at day seven and recovery by day 21 are expected and consistent with current collateral research, the alternative pathways and responses of the ACCs and PECs in the Balb/C strain in particular have, previously, been largely unexplored (8, 25, 48, 52, 63).

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 an occlusion and reach downstream tissues. Further understanding of the mechanisms of arterialized collateral capillary development may expose specific elements that are involved; these elements could be exogenously introduced to elicit or quicken the collateral development process. The sooner blood returns to an ischemic area, the less time the tissues lack oxygen, nutrients, and the potential for necrosis, overall improving patient prognosis (43).

REFERENCES

1. Ballard KD, Tschakovsky ME, Zaleski AL, Polk DM, Thompson PD, Kiernan FJ, Parker B a. Redundant vasodilator pathways underlying radial artery flow-mediated dilation are preserved in healthy aging. *J. Aging Res.* 2014: 1–8, 2014.

2. Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol. Rev.* 84: 869–901, 2004.

3. Bech Laursen J, Boesgaard S, Enghusen Poulsen H, Aldershvile J. Nitrate tolerance impairs nitric oxide-mediated vasodilation in vivo. *Cardiovasc. Res.* 31: 814–819, 1996.

4. Boger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease [see comments]. *Circulation* 95: 2068–2074, 1997.

5. Boron WF, Boulpaep EL. *Medical physiology: a cellular and molecular approach*. 2009.

6. Brandes RP, Schmitz-Winnenthal FH, Félétou M, Gödecke a, Huang PL, Vanhoutte PM, Fleming I, Busse R. An endothelium-derived hyperpolarizing factor distinct from NO and prostacyclin is a major endothelium-dependent vasodilator in resistance vessels of wild-type and endothelial NO synthase knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* 97: 9747–52, 2000.

7. Busse R, Edwards G, Félétou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: Bringing the concepts together. *Trends Pharmacol. Sci.* 23: 374–380, 2002.

8. Bynum A. Impact of Collateral Enlargement on Smooth Muscle Phenotype. *Calif. Polytech. State Univ.* California Polytechnic State University. 2011.

9. Cardinal TR, Struthers KR, Kesler TJ, Yocum MD, Kurjiaka DT, Hoying JB. Chronic hindlimb ischemia impairs functional vasodilation and vascular reactivity in mouse feed arteries. *Front. Physiol.* 2: 91, 2011.

10. CDC. Peripheral Arterial Disease (PAD) Fact Sheet. *Centers Dis. Control Prev.* 2011.

11. Chalothorn D, Clayton JA, Zhang H, Pomp D, Faber JE. Collateral density, remodeling, and VEGF-A expression differ widely between mouse strains. *Physiol. Genomics* 30: 179–191, 2007.

12. Chalothorn D, Faber JE. Strain-dependent variation in collateral circulatory function in mouse hindlimb. *Physiol. Genomics* 42: 469–479, 2010.

13. Cutts J, McGabhann F, Cardinal TR. Functional Vasodilation of Arterialized Capillaries Is Impaired in the Spinotrapezius. California Polytechnic State University, San Luis Obispo. [date unknown].

14. Darius H, Lefer AM, Leprán I, Smith JB. In vivo interaction of prostacyclin with an inhibitor of cyclic nucleotide phosphodiesterase, HL 725. *Br. J. Pharmacol.* 84: 735–741, 1985.

15. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation* 109: III27–32, 2004.

16. Davis GE, Bayless KJ, Mavila A. Molecular basis of endothelial cell morphogenesis in three-dimensional extracellular matrices. *Anat. Rec.* 268: 252–275, 2002.

17. Duza T, Sarelius IH. Conducted dilations initiated by purines in arterioles are endothelium dependent and require endothelial Ca2+. *Am. J. Physiol. Heart Circ. Physiol.* 285: H26–H37, 2003.

18. Emerson GG, Segal SS. Electrical coupling between endothelial cells and smooth muscle cells in hamster feed arteries: role in vasomotor control. *Circ. Res.* 87: 474–479, 2000.

19. Félétou M, Vanhoutte PM. Endothelium-derived hyperpolarizing factor: where are we now? *Arterioscler. Thromb. Vasc. Biol.* 26: 1215–25, 2006.

20. Félétou M. The Endothelium, Part I: Multiple Functions of the Endothelial Cells -- Focus on Endothelium-Derived Vasoactive Mediators. *Colloq. Ser. Integr. Syst. Physiol. From Mol. to Funct.* 3: 1–306, 2011.

21. Feringa HHH, van Waning VH, Bax JJ, Elhendy A, Boersma E, Schouten O, Galal W, Vidakovic R V, Tangelder MJ, Poldermans D. Cardioprotective medication is associated with improved survival in patients with peripheral arterial disease. *J. Am. Coll. Cardiol.* 47: 1182–1187, 2006.

22. Fisher SA. Vascular smooth muscle phenotypic diversity and function. *Physiol. Genomics* 42A: 169–187, 2010.

23. Fujita M, Yamanishi K, Inoko M, Miwa K. Preferential Dilation of Recipient Coronary Arteries of the Collateral Circulation by Intracoronary Administration of Nitroglycerin. 2: 631–5, 1994.

24. Fujiwara H, Wake Y, Hashikawa-Hobara N, Makino K, Takatori S, Zamami Y, Kitamura Y, Kawasaki H. Endothelium-Derived Relaxing Factor–Mediated Vasodilation in Mouse Mesenteric Vascular Beds. *J. Pharmacol. Sci.* 118: 373–381, 2012.

25. Gabhann F Mac, Peirce SM. Collateral capillary arterialization following arteriolar ligation in murine skeletal muscle. *Microcirculation* 17: 333–347, 2010.

26. Gallagher R. The impact of outward remodeling on vasodilation in skeletal muscle resistance arteries. *Calif. Polytech. State Univ.* California Polytechnic State University. 2012.

27. Go J, Cardinal TR. Development of a Blood Flow Measurement Protocol Using Particle Image Velocimetry. California Polytechnic State University, San Luis Obispo. 2012.

28. Hajjar I, Kotchen JM, Kotchen T a. Hypertension: trends in prevalence, incidence, and control. *Annu. Rev. Public Health* 27: 465–90, 2006.

29. Handschin C, Spiegelman BM. The role of exercise and PGC1alpha in inflammation and chronic disease. *Nature* 454: 463, 2008.

30. Hattenbach LO, Allers A, Klais C, Koch F, Hecker M. L-Arginine-nitric oxide pathway-related metabolites in the aqueous humor of diabetic patients. *Invest. Ophthalmol. Vis. Sci.* 41: 213–217, 2000.

31. Hill CE. Long distance conduction of vasodilation: a passive or regenerative process? *Microcirculation* 19: 379–90, 2012.

32. Hool LC. Can Integrins Integrate Vascular Myogenic Responses? *Circ. Res.* 90: 371–373, 2002.

33. Van Huysse JW, Amin MS, Yang B, Leenen FHH. Salt-induced hypertension in a mouse model of Liddle syndrome is mediated by epithelial sodium channels in the brain. *Hypertension* 60: 691–6, 2012.

34. Johnson BD, Wilson LE, Zhan WZ, Watchko JF. Contractile properties of the developing with myosin heavy chain phenotype diaphragm correlate. *J. Appl. Physiol.* 77: 481–7, 1994.

35. Katz SD, Biasucci L, Sabba C, Strom JA, Jondeau G, Galvao M, Solomon S, Nikolic SD, Forman R, LeJemtel TH. Impaired endothelium-mediated vasodilation in the peripheral vasculature of patients with congestive heart failure. *J. Am. Coll. Cardiol.* 19: 918–25, 1992.

36. Kellogg DL, Zhao JL, Coey U, Green J V. Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin. *J. Appl. Physiol.* 98: 629–32, 2005.

37. Kelsall CJ, Brown MD, Hudlicka O. Alterations in reactivity of small arterioles in rat skeletal muscle as a result of chronic ischaemia. *J. Vasc. Res.* 38: 212–218, 2001.

38. Klabunde R. *Cardiovascular physiology concepts*. Lippincott Williams & Wilkins, 2011.

39. Kobayashi H, DeBusk LM, Babichev YO, Dumont DJ, Lin PC. Hepatocyte growth factor mediates angiopoietin-induced smooth muscle cell recruitment. *Blood* 108: 1260–6, 2006.

40. Koeppen BM, Stanton BA. *Berne & Levy Physiology*. Elsevier Health Sciences, 2009.

41. Long KM, Kirby R. An update on cardiovascular adrenergic receptor physiology and potential pharmacological applications in veterinary critical care. *J. Vet. Emerg. Crit. Care* 18: 2–25, 2008.

42. Martinez-Lemus LA, Hill MA, Meininger GA. The plastic nature of the vascular wall: a continuum of remodeling events contributing to control of arteriolar diameter and structure. *Physiology (Bethesda).* 24: 45–57, 2009.

43. Meier P, Gloekler S, Zbinden R, Beckh S, De Marchi SF, Zbinden S, Wustmann K, Billinger M, Vogel R, Cook S, Wenaweser P, Togni M, Windecker S, Meier B, Seiler C. Beneficial effect of recruitable collaterals: A 10-year follow-up study in patients with stable coronary artery disease undergoing quantitative collateral measurements. *Circulation* 116: 975–983, 2007.

44. Muller JM, Chilian WM, Davis MJ. Integrin signaling transduces shear stress--dependent vasodilation of coronary arterioles. *Circ. Res.* 80: 320–326, 1997.

45. Murrant CL, Sarelius IH. Multiple dilator pathways in skeletal muscle contraction-induced arteriolar dilations. *Am. J. Physiol. Integr. Comp. Physiol.* 282: R969–78, 2002.

46. Needleman P, Key SL, Denny SE, Isakson PC, Marshall GR. Mechanism and modification of bradykinin-induced coronary vasodilation. *Proc. Natl. Acad. Sci. U. S. A.* 72: 2060–3, 1975.

47. NIH. What is Peripheral Arterial Disease? *Natl. Institutes Heal.* 2011.

48. Peirce S, Skalak TC. Microvascular Remodeling: A Complex Continuum Spanning Angiogenesis to Arteriogenesis. *Microcirculation* 10: 99–111, 2003.

49. Peng X, Wang J, Lassance-Soares RM, Najafi AH, Sood S, Aghili N, Alderman LO, Panza JA, Faber JE, Wang S, Epstein SE, Burnett MS. Gender differences

affect blood flow recovery in a mouse model of hindlimb ischemia. *Am. J. Physiol. Heart Circ. Physiol.* 300: H2027–H2034, 2011.

50. Piascik MT, Perez DM. Alpha1-Adrenergic Receptors : New Insights and Directions. *Perspect. Pharmacol.* 298: 403–410, 2001.

51. Piek J, Liebergen R van, Koch K. Pharmacological Modulation of the Human Collateral Vascular Resistance in Acute and Chronic Coronary Occlusion Assessed by Intracoronary Blood Flow Velocity Analysis in an Angioplasty Model. *Circulation* 96: 106–115, 1997.

52. Prior BM, Lloyd PG, Ren J, Li H, Yang HT, Laughlin MH, Terjung RL. Time course of changes in collateral blood flow and isolated vessel size and gene expression after femoral artery occlusion in rats. *Am. J. Physiol. Heart Circ. Physiol.* 287: H2434–47, 2004.

53. Qiagen. Sample & Assay Technologies: ENOS Signaling. 1999.

54. Ratcliffe HA. Intermittent Claudication: A Review. *Postgrad. Med. J.* 26: 436, 1950.

55. Robbins S, Kumar V, Abbas AK, Aster JC. *Robbins basic pathology*. Elsevier Health Sciences, 2012.

56. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol. Rev.* 82: 131–85, 2002.

57. Rondelli CM, Szasz IT, Kayal A, Thakali K, Watson RE, Rovner AS, Eddinger TJ, Fink GD, Watts SW. Preferential myosin heavy chain isoform B expression may contribute to the faster velocity of contraction in veins versus arteries. *J. Vasc. Res.* 44: 264–272, 2007.

58. Royen N van, Piek JJ, Buschmann I, Hoefer I, Voskuil M, Schaper W. Stimulation of arteriogenesis; a new concept for the treatment of arterial occlusive disease. *Cardiovasc. Res.* 49: 543–553, 2001.

59. Russell JC, Proctor SD. Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. *Cardiovasc. Pathol.* 15: 318–30, 2006.

60. SAGE Labs. Knockout Models: C57Bl/6 Inbred Mouse. *SAGE Labs* 2013.

61. Saito Y, Eraslan A, Hester RL. Role of Endothelium-Derived Relaxing Factors in Arteriolar Dilation During Muscle Contraction Elicited by Electrical Field Stimulation. *Microcirculation* 1: 195–201, 1994.

62. Schaper W. Collateral vessels reduce mortality. *Eur. Heart J.* 33: 564–6, 2012.

63. Schirmer SH, Nooijen FC van, Piek JJ, Royen N van. Stimulation of collateral artery growth: travelling further down the road to clinical application. *Heart* 95: 191–7, 2009.

64. Scott-Okafor HR, Silver KK, Parker J, Almy-Albert T, Gardner AW. *Lower extremity strength deficits in peripheral arterial occlusive disease patients with intermittent claudication.* 2001.

65. Seiler C. The human coronary collateral circulation. *Eur. J. Clin. Invest.* 40: 465–76, 2010.

66. Shaw JE, Sicree R a, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res. Clin. Pract.* 87: 4–14, 2010.

67. Sheetz MP, Felsenfeld DP, Galbraith CG. Cell migration: regulation of force on extracellular-matrix-integrin complexes. *Trends Cell Biol.* 8: 51–4, 1998.

68. Stapor PC, Azimi MS, Ahsan T, Murfee WL. An angiogenesis model for investigating multicellular interactions across intact microvascular networks. *Am. J. Physiol. Heart Circ. Physiol.* 304: H235–45, 2013.

69. Stapor PC, Murfee WL. Spatiotemporal distribution of neurovascular alignment in remodeling adult rat mesentery microvascular networks. *J. Vasc. Res.* 49: 299–308, 2012.

70. Straub AC, Johnstone SR, Heberlein KR, Rizzo MJ, Best AK, Boitano S, Isakson BE. Site-specific connexin phosphorylation is associated with reduced heterocellular communication between smooth muscle and endothelium. *J. Vasc. Res.* 47: 277–286, 2010.

71. Swierkosz TA, Mitchell JA, Warner TD, Botting RM, Vane JR. Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids. *Br. J. Pharmacol.* 114: 1335–1342, 1995.

72. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat. Rev. Immunol.* 10: 36–46, 2010.

73. Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita a. Importance of endothelium-derived hyperpolarizing factor in human arteries. *J. Clin. Invest.* 100: 2793–9, 1997.

74. Waltenberger J. Impaired collateral vessel development in diabetes: potential cellular mechanisms and therapeutic implications. *Cardiovasc. Res.* 49: 554–560, 2001.

75. Watanabe H, Tran QK, Takeuchi K, Fukao M, Liu MY, Kanno M, Hayashi T, Iguchi a, Seto M, Ohashi K. Myosin light-chain kinase regulates endothelial calcium entry and endothelium-dependent vasodilation. *FASEB J.* 15: 282–4, 2001.

76. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature* 407: 242–248, 2000.

77. Zannad F, Stough WG, Mahfoud F, Bakris GL, Kjeldsen SE, Kieval RS, Haller H, Yared N, De Ferrari GM, Piña IL, Stein K, Azizi M. Design Considerations for Clinical Trials of Autonomic Modulation Therapies Targeting Hypertension and Heart Failure. *Hypertension* (October 27, 2014). doi: 10.1161/HYPERTENSIONAHA.114.04057.

78. Zaragoza C, Gomez-Guerrero C, Martin-Ventura JL, Blanco-Colio L, Lavin B, Mallavia B, Tarin C, Mas S, Ortiz A, Egido J. Animal models of cardiovascular diseases. *J. Biomed. Biotechnol.* 2011: 497841–497813, 2011.

79. Zbinden S, Clavijo LC, Kantor B, Morsli H, Cortes GA, Andrews JA, Jang GJ, Burnett MS, Epstein SE. Interanimal variability in preexisting collaterals is a major factor determining outcome in experimental angiogenesis trials. *Am. J. Physiol. - Hear. Circ. Physiol.* 292: H1891–7, 2007.

80. Zhang RL, Chopp M, Chen H, Garcia JH. Temporal profile of ischemic tissue damage, neutrophil response, and vascular plugging following permanent and transient (2H) middle cerebral artery occlusion in the rat. *J. Neurol. Sci.* 125: 3–10, 1994.

APPENDIX A

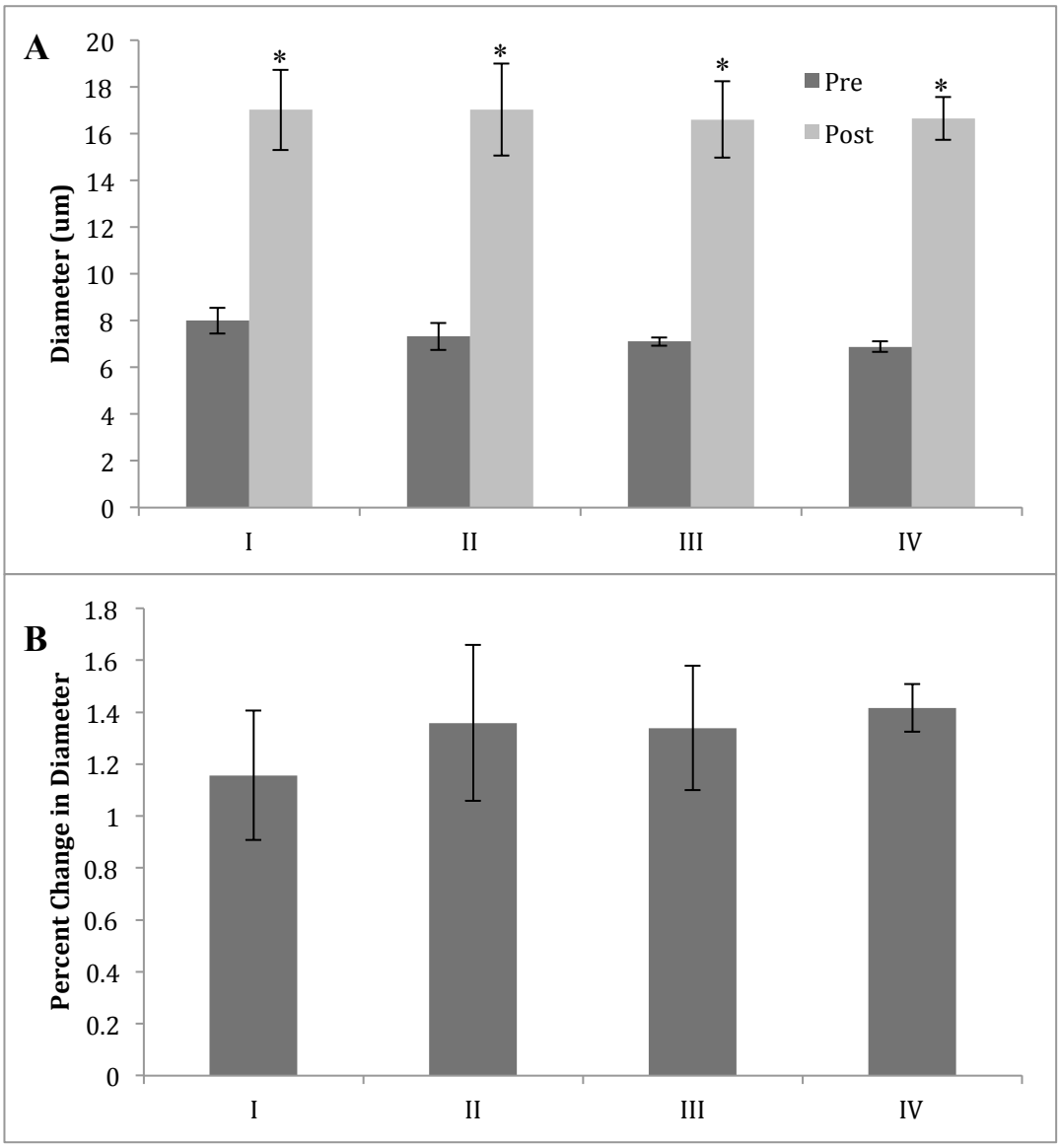

Figure 20. Responses to repetitive functional vasodilation in unoperated animals. A) Arteriole diameters pre and post stimulation for four repetitions of 90s electrode-induced contraction with 30 minutes between each repetition. **B)** Percent changes of diameters at each stimulation; * indicates $p < 0.05$.

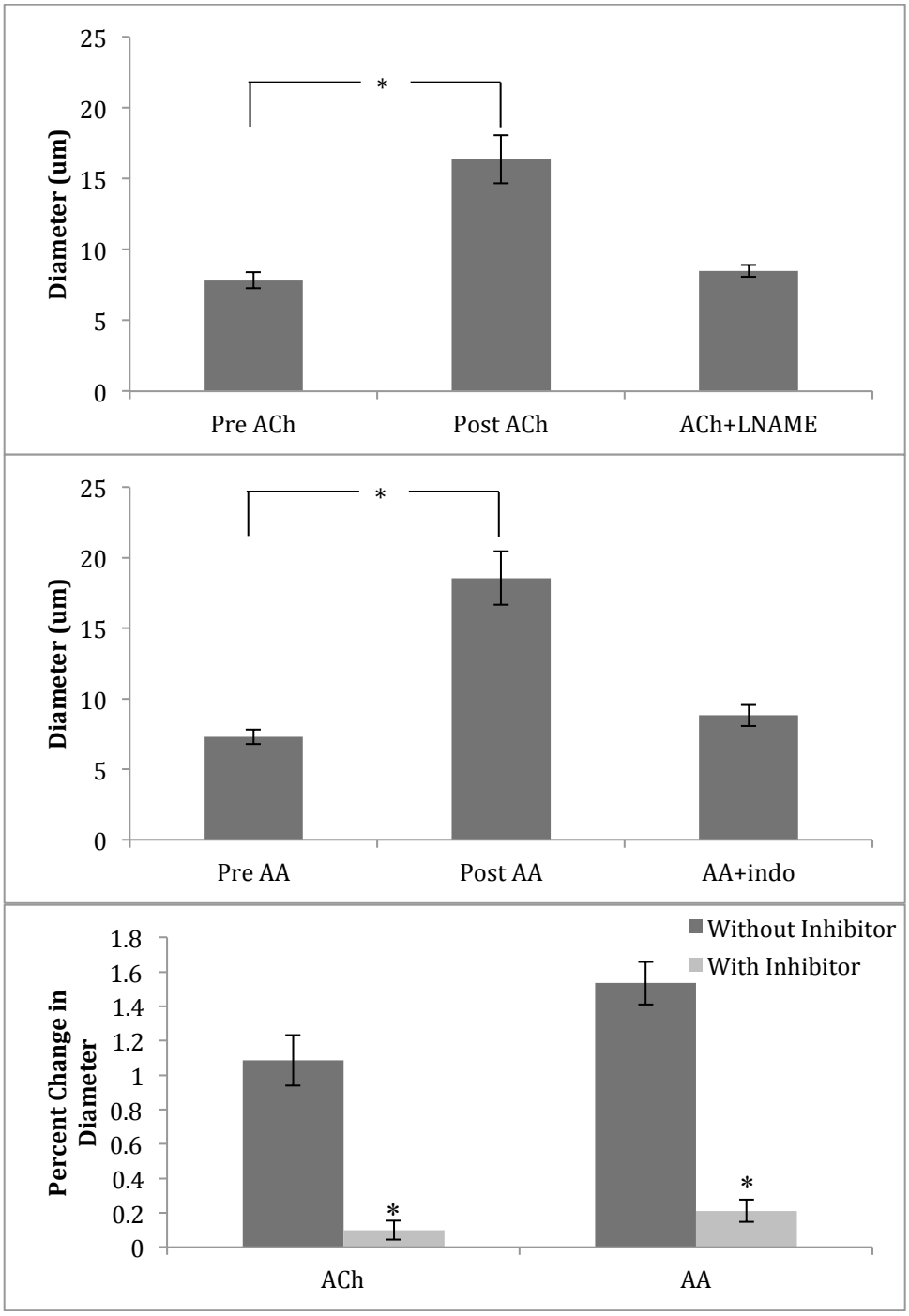

Figure 21. Efficacy of vasodilation inhibitors in unoperated animals. Arteriole diameters (μm) pre and post exposure to vasodilators, as well as post exposure to vasodilators in combination with vasodilation inhibitors using 10^{-5} Molar acetylcholine (ACh) as a dilator and $2x10^{-5}$ L-NAME as an inhibitor **(A)**, and using $2x10^{-4}$ Molar arachidonic acid (AA) as a dilator and $2x10^{-4}$ Molar indomethacin as an inhibitor **(B)**. **C)** Percent changes in diameter in response to the respective dilators and inhibitors; * indicates $p < 0.05$.

APPENDIX B

Date _____ **Spinotrapezius Ligation** Initials _____

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

Materials
Instruments
____1. Standard Pattern Forceps (1)
____2. Iris Scissors (1)
____3. S & T (2)
____4. 5/45 (1)
____5. Dumont #7's (1)
____6. Microdissection scissors (1)
____7. Needle Holders (1)

Pre-sterilize in autoclave
____8. Cotton gauze (4)
____9. Cotton swabs (4)
____10. 6.0 silk suture (2x1 in)
____11. Surgical Drape

Obtained in surgery suite
____12. Petri dish w/ sterile saline
____13. Gloves
____14. Sterile 7-0 prolene suture
____15. FST heat pad w/ rectal probe
____16. heat pad
____17. Recovery bin and weigh boat
____18. Depilatory cream
____19. non-sterile cotton swabs
____20. non-sterile cotton gauze
____21. Isolation mask & cap
____22. Analgesic (buprenorphine)

Surgery Preparation
____23. Spray surgery area with Nolvasan
____24. Weigh animal in weight boat
____25. Place animal in anesthesia box
____26. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 $l \cdot min^{-1}$
____27. Anesthetize animal w/ 5% isoflurane
____28. Reduce flow rate to 0.5-1.0 $l \cdot min^{-1}$ and the isoflurane to 1-3%
____29. Lay animal prone with nose in nose-cone
____30. Remove hair on anterior dorsal aspect of the animal with clippers and depilatory cream.
____31. Apply vetinary ointment to eyes to avoud drying during the procedure
____32. Give the animal subcutaneous injection of buprenorphine
____33. Lay animal prone on circulating heat pad (w/ 4x4 on top) w/ nose in nose-cone
____34. Insert rectal probe and set thermo-controller to 35°C
____35. Insert rectal probe and set thermo-controller to 35 ° C
____36. Adjust focus, lighting, and center mouse
____37. Don isolation mask, bonnet, and scrubs
____38. Open sterile surgical instrument pack
____39. Open sterile pack and dump in surgical instrument field
____40. Place sterile petri dish in surgical field and fill with sterile saline
____41. Don sterile gloves
____42. Transfer the content of sterile pack to surgical instrument sterile field (leave 1 4x4 on bench for non-sterile hands)

Surgery
____43. Make 5mm incision parallel to spine at intersection of fat pad and muscle
____44. Dissect skin overlying the spinotrapezius muscle
____45. Search for lateral feed artery vein pair jumping from the underlying fat pad to the spinotrapezius
____46. Blunt dissect fat pad to reveal arteriole-venule pair, using lidocaine as a vasodilator to aid in visualization
____47. Blunt dissect arteriole away from venule
____48. Pass a separated strand from 6-0 silk suture below the arteriole and tie off
____49. Repeat again about 1mm above previous ligature
____50. Cut arteriole between the two ligatures with micro dissection scissors
____51. Suture incision with 7-0 prolene suture
____52. Repeat for contralateral side to perform sham, but do not tie off ligature

Post-Surgical
____53. Give the animal a subcutaneous injection of buprenorphine
____54. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover
____55. Turn the flow meter down to 0, turn off the isoflurane, and close the oxygen cylinder
____56. Indicate surgery on cage card.

Notes
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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 50ºC
____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

Date _____ **Intravital Microscopy with Superfusion** Initials _____

Post-Experiment

____59. Cervical dislocation to euthanize animal
____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
Reagent 1 Resting Diameter _____
Reagent 1 volume & concentration _____
Reagent 1 Dose 1 diameter _____
Reagent 2 Resting Diameter _____
Reagent 2 volume & concentration _____
Reagent 2 diameter _____
Reagent 3 Resting Diameter _____
Reagent 3 volume & concentration _____
Reagent 3 diameter _____

Limb 2

Left or Right
Reagent 1 Resting Diameter _____
Reagent 1 volume & concentration _____
Reagent 1 Dose 1 diameter _____
Reagent 2 Resting Diameter _____
Reagent 2 volume & concentration _____
Reagent 2 diameter _____
Reagent 3 Resting Diameter _____
Reagent 3 volume & concentration _____
Reagent 3 diameter _____

Notes

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