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Exogenous Thrombin Delivery Promotes Collateral Capillary Arterialization and Tissue Reperfusion in the Murine Spinotrapezius Muscle Ischemia Model

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Abstract

Objective—We examined the effects of exogenously delivered thrombin on cell recruitment in skeletal muscle and the formation of new collateral arterioles in the microvasculature in response to ligation-induced ischemia.

Methods—Thrombin or vehicle was locally applied to both ligated and non-operated Balb/c spinotrapezius muscles which were harvested after three or seven days, imaged using confocal microscopy, and analyzed.

Results—Thrombin treatment resulted in accelerated arterialization of collateral capillaries and accelerated tissue reperfusion in ischemic muscles. Uninjured muscle treated with thrombin displayed increased vascular cell adhesion molecule 1 expression on arteriole and venule endothelium, increased expression of smooth muscle α-actin on capillary-sized vessels, increased infiltration by $CD11b^+$ leukocytes, and mast cell infiltration and degranulation.

Conclusion—Exogenous delivery of thrombin enhances microvascular collateral development in response to ischemic insult and accelerates tissue reperfusion. Elicited responses from multiple cell types likely contribute to these effects.

Keywords

thrombin; arteriogenesis; collateral vessel; ischemia

Introduction

Collateral arteries are high conductance artery-to-artery anastomoses that confer protection against obstructive vascular disease[43, 47]. Evidence in animal models and humans suggests that individual variability exists in the number of native collateral arteries, and this variability renders some individuals more susceptible to ischemic diseases than others[8, 36]. A treatment to induce the formation of new collateral arteries, particularly in diseased or aged tissues that lack the propensity to form new collateral vessels could be highly effective in conferring protection against ischemia for patients suffering from peripheral arterial disease and heart disease.

Thrombin, a serine protease involved in the coagulation cascade, has mitogenic effects on vascular cells[23, 24, 35] and is a known inducer of inflammation (for review see[44]). In cultured

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Disclosures
None.

vascular endothelial cells, thrombin has been demonstrated to upregulate expression of E-selectin[25], ICAM-1[26, 50], IL-8[25], MCP-1[12, 55], PDGF[15], P-selectin[50], and VCAM-1[26]. In cultured vascular smooth muscle cells, thrombin has been demonstrated to upregulate bFGF[13], HIF-1[19], MCP-1[12, 55], TGF-β1[3], and VEGF[7]. In addition, thrombin has been shown to activate some leukocyte populations[5, 39] and to induce MCP-1 production in monocytes[12].

It was recently shown that exogenous intramuscular delivery of thrombin promoted collateral development and reperfusion in a rabbit hind-limb model of ischemia[27]. This study examined large collateral arteriole (>500 μm diameter) formation and reperfusion after injury but did not examine the microcirculation at the capillary level or the effects of thrombin on microvasculature in healthy non-ischemic muscle.

In the Balb/c mouse spinotrapezius, collateral capillaries (capillaries connecting separate arteriolar watersheds[31]) undergo arterialization in response to ischemic injury, whereby they undergo diameter increase and recruit SMA positive mural cells, thus becoming new arterioles[31]. We hypothesized that thrombin would enhance capillary arterialization in the mouse spinotrapezius ligation model of ischemia. Most studies examining the effects of thrombin on various cell types have been performed *in vitro*. This paper builds upon that literature by examining for the first time the effects of exogenous thrombin on the formation of new collateral arterioles from capillaries in response to ischemia, its effect on reperfusion in the spinotrapezius following ischemic injury, and its effects on the recruitment of select leukocyte populations and perivascular mural cells *in vivo*.

Materials and Methods

Mice and study overview

All surgical protocols were approved by the Animal Care and Use Committee of the University of Virginia. Male BALB/cAnNHsd (Harlan) mice (age 8–12 weeks) were utilized because of their tendency to have unconnected arborized arteriolar watersheds which form arterialized collaterals in response to ligation-induced ischemia[31]. Female mice were included in the 7-day ligation groups and were distributed equally across treatments; we have previously shown that both genders have similar innate microvascular network architectures and exhibit comparable abilities to undergo microvascular remodeling[31].

To establish the effects of thrombin on the arteriogenesis of collateral capillaries in response to ischemic injury, ligation of a main feeding arteriole was performed on a single spinotrapezius muscle per animal as described below and previously published[4, 31], and thrombin or vehicle control was perfused over the muscle before suturing.

To establish the effects of thrombin in the absence of both ischemia and inflammation induced by ligation surgery, thrombin or vehicle control was injected subcutaneously over a single spinotrapezius per animal posterior to the fat pad.

Right and left spinotrapezius muscles were harvested after either three or seven days. Manipulated muscles were stained with immunofluorescent markers for vascular structures and leukocytes, whole-mounted, imaged using a confocal microscope, and analyzed as described below. Unmanipulated muscles were used for unstained and fluorescence-minus-one controls. Fluorescence-minus-one controls lacked one conjugated antibody from the group of conjugated antibodies used to label test specimens, thus allowing us to determine levels of autofluorescence.

Ligation Surgery

All mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine/xylazine/atropine (60/4/0.2 mg/kg). Ligation surgeries were performed as previously described[4, 31]. Briefly, a small incision was made on the dorsum above the lateral edge of the right spinotrapezius at the edge of the fat pad. The fascia was separated from the top of the muscle and the fat pad moved before isolating an anatomically reproducible feeding arteriole entering the muscle from below. This feeding arteriole was ligated with 10-0 non-absorbable suture in two places and cut. The fat pad and fascia were moved back into position and the skin incision sutured with 8-0 non-absorbable suture.

Thrombin Delivery

Bovine thrombin (Sigma-Aldrich, St. Luis, MO) was diluted in sterile saline to a concentration of 1 NIH unit /μl. Immediately following ligation, 1 μl per gram of body weight was perfused over the spinotrapezius muscle and under its lateral edge using a 27-gauge insulin syringe prior to the fascia and fat pad being returned to position and the skin incision being sutured. Controls received sterile saline vehicle only. Non-operated mice received identical thrombin or vehicle dose by subcutaneous injection over the caudal portion of the right spinotrapezius muscle posterior to the fat pad.

Perfusion Labeling

To visualize loss of perfusion in ligated muscles, anesthetized mice were administered an intra-jugular injection of fluorescently-labeled isolectin in phosphate buffered saline (IB4-Alexa647, Sigma, St. Louis, MO) ten minutes before euthanization. The jugular was surgically exposed before a volume of 250 μl at a concentration of 0.1 mg/ml was delivered slowly using a 27-guage insulin syringe. A solution of adenosine (70mg/L) in phosphate buffered saline was applied topically to the spinotrapezius prior to injection to dilate the blood vessels. We examined perfusion three days post-surgery since we have previously shown it to be fully restored by seven days post-surgery[31].

Harvest and Immunostaining

Mice were euthanized either three or seven days post-treatment by overdose i.p. injection of ketamine/xylazine/atropine (120/8/0.4 mg/kg). These time points were chosen based on considerations for monocyte recruitment during arteriogenesis[2] and muscle reperfusion following ligation-induced ischemia[31]. The vasculature was then flushed with an intracardiac infusion of 0.9% heparanized saline followed by an intracardiac infusion of 4% paraformaldehyde in phosphate buffered saline. Spinotrapezius muscles were stripped of fascia, undermined, excised, and stored in cold phosphate buffered saline prior to staining. In solution, the muscles were washed, permeabilized in 0.1% saponin (Sigma-Aldrich, St. Luis, MO), and stained for a combination of smooth muscle α-actin (SMA) [mural cell stain, IA4-Cy3 (Sigma, St. Luis, MO), 1:300], isolectin [endothelial cell stain, IB4-Alexa647 (Invitrogen, Carlsbad, CA), 1:200], CD11b [leukocyte stain, M1/70.15-Alexa488 (Invitrogen, Carlsbad, CA), 1:300], and VCAM-1 [activated endothelial cell stain, 429 (MVCAM.A)-Alexa647 (Biolegend, San Diego, CA),1:200] overnight at 4°C. All muscles analyzed for CD11b+ leukocytes were blocked with 5% normal mouse and 5% normal rat serum (eBioscience, San Diego, CA) prior to immunostaining.

Determination of Presence of Collateral Capillary Arterialization

After staining, all ligated muscles were first examined for collateral vessels undergoing diameter expansion in areas bordering the ligated arteriolar watershed (Figure 1A). Muscles lacking this feature invariably had one to two large arteriole collaterals supplying redundant blood flow to the ligated arteriolar watershed (Figure 1B). Based on their relatively large

diameters (38–62 μm), their apparent remodeling in the form of increased tortuosity compared to neighboring arterioles, and the lack of collateral vessels undergoing diameter expansion on the borders of the ligated arteriolar watershed, we concluded these large arteriole collaterals to be preexisting. Muscles which lacked evidence of collateral capillary arterialization were excluded from the analyses of this process.

Imaging and Data Analysis

Digital images of immunolabeled whole-mount spinotrapezius tissue were acquired using confocal microscopy (Nikon, Model TE200-E2; 4x, 10x, 20X, and 60X objectives). Analyses of the confocal images were conducted using ImageJ (NIH, Bethesda, MD) imaging software to quantify all lengths and diameters.

The largest-diameter collateral in each muscle was located by visual inspection of isolectin and SMA staining. 100X and 200X volume renders from full-vessel-thickness Z-stacks using 10X and 20X objectives and a 2 μm Z-step size were generated. In cases of sparse SMA+ mural cell coverage, collateral diameters were measured at the mid-point between opposing arteriolar watershed arteriole terminals using isolectin staining. In cases of complete or near-complete SMA+ mural cell coverage, collateral diameters were measured at their midpoints using isolectin staining where the collateral ends were defined as the first branching in each direction to exceed 10 μm in diameter and have SMA+ mural cell coverage.

To quantify perfusion, montages of the ligated arteriolar watershed were constructed from 40X images. These montages were quantified for visible SMA length and visible isolectin length and a ratio of the two generated.

To quantify CD11b+ leukocyte recruitment, 100X volume renders from full-muscle-thickness Z-stacks using a 10X objective and 2 μm Z-step size were generated in areas of the muscle displaying collateral capillary arterialization. These images were thresholded to identical pixel-intensity values to eliminate background then converted to black and white. Finally, the images were analyzed for percent MAC-1 positive pixels using Matlab (MathWorks, Natick, MA).

To quantify VCAM-1 levels, 100X volume renders from full-muscle-thickness Z-stacks using a 10X objective and 2 μm Z-step size were generated from areas in the caudal spinotrapezius containing an arteriole/venule pair. These images were thresholded to identical pixel-intensity values to eliminate background then converted to black and white. Finally, the images were analyzed for percent VCAM-1 positive pixels using Matlab (MathWorks, Natick, MA).

In non-operated muscles, areas of capillary-sized vessels expressing SMA at arteriole terminals were located by visual inspection. 100X volume renders were generated from full-muscle-thickness Z-stacks using a 10X objective and 2 μm Z-step size. Arteriole terminals in control muscles were then imaged in anatomically matched locations. The pixel lengths of SMA coverage on vessels 10 μm and less in diameter were measured and added to generate a sum total for each image.

In ligated muscles, regions of the largest developing collateral which were covered in SMA+ mural cells around the entire visible vessel circumference were measured for SMA length and these lengths summed. Then a ratio of this SMA length to the length of the entire collateral was generated. When measuring total length, collateral ends were defined as the first branching in each direction to exceed 10 μm in diameter and have SMA+ mural cell coverage. The same 100X volume renders used for diameter analyses were used.

To quantify the number of collateral capillaries undergoing arterialization after three days in 100X fields containing the largest diameter collateral, a line was drawn perpendicular to the remodeling collaterals midway between the opposing arteriole terminals. In cases of newly formed collateral arterioles displaying complete SMA+ mural cell coverage, the line was placed so it intersected the midpoints of the collaterals where the collateral ends were defined as the first branching in each direction to exceed 10 μm in diameter and have SMA+ mural cell coverage. Each vessel in question was then examined at the portion where the line intersected it for SMA expression and a sum generated for the total number of SMA+ intersections per field of view.

Statistical Analysis

Statistical analysis was performed using SigmaStat 3.1 software (Systat Software, Inc., San Jose, CA). Significance of differences was tested using 2-way ANOVA, unpaired Student's t-test, or the Mann-Whitney Rank Sum test as appropriate. Statistical significance was asserted at $p<0.05$.

Results

Thrombin activates the microvascular endothelium

Thrombin-treated, non-operated muscles displayed significant up-regulation of VCAM-1 in both arterioles and venules compared to vehicle-treated, non-operated controls three days post-injection (Figure 2). Thrombin treatment increased expression by more than 10-fold after three days as estimated by positive pixel counts.

Unpublished data (not shown) from our group indicates that in ligated muscles lacking exogenous thrombin, VCAM-1 expression is similarly upregulated on arterioles and venules adjacent to the ligated arteriolar watershed at one day post-surgery, but this upregulation is lost by two days post-surgery.

Thrombin promotes leukocyte infiltration

Vehicle-treated, non-operated muscles lacked substantial infiltration of CD11b+ cells (Figure 3A&B). CD11b+ leukocytes were significantly increased in thrombin-treated, non-operated muscles three days post-injection (Figure 3A&C). In ligated muscles, there was no significant difference ($p=0.48$) in CD11b+ cell infiltration between treatments (Figure 3D, E, &F). CD11b+ cells were concentrated in the first 50 μm of the muscle as measured from the superficial surface, which is where collateral capillaries undergoing arterialization and areas of capillaries expressing SMA were predominately observed.

Thrombin-treated groups exhibited substantial mast cell infiltration which was not observed in vehicle-treated groups (Figure 3G). Mast cells were often closely associated with arterioles and venules and were undergoing degranulation in some instances (Figure 3H). These cells were visualized in non-blocked muscle by non-specific binding of anti-SMA (IA4-Cy3) and their presence was further confirmed by toluidine blue staining in sectioned, paraffin-embedded, muscles (data not shown).

Thrombin promotes SMA+ mural cell investment

Thrombin treatment in non-operated muscles resulted in capillary-sized vessels displaying increased SMA coverage compared to vehicle-treated non-operated muscles. This effect was most prominent in the thin posterior-most portion of the spinotrapezius. Comparisons of same-sized fields of view matched for anatomical location showed an approximately 4-fold increase in SMA pixel length on vessels with diameters of 10 μm and less in thrombin-treated muscles three days post-injection versus those receiving vehicle (Figure 4A, B, &C).

At the border between SMA+ arterioles and SMA- capillaries in these areas, individual SMA+ cells in contact with the endothelium were visible in thrombin-treated muscles but not in vehicle-treated (Figure 4D & E).

In ligated muscles, thrombin treatment resulted in increased average mural cell investment on the largest collaterals undergoing arterialization after three days compared to vehicle-treated. This difference did not reach statistical significance ($p = 0.08$) but only the thrombin-treated group contained collaterals exhibiting total SMA+ mural cell coverage after only three days (Figure 4F, G, & H). Remodeling collaterals of both treatment groups displayed full SMA+ mural cell coverage by seven days post-surgery.

Thrombin accelerates collateral capillary arterialization in the presence of ischemia

In ligated muscles where microvascular remodeling was observed in collateral vessels connecting to the ligated arteriolar watershed, thrombin treatment significantly increased maximum collateral diameters achieved after both three and seven days, relative to vehicle control. Maximum diameters achieved increased significantly with time in both vehicle-treated and thrombin-treated groups (Figure 5A).

Thrombin-treated groups possessed fewer parallel remodeling collaterals in 100X fields of view containing the largest-diameter collateral as measured by SMA+ intersections. The number of these intersections increased significantly between days three and seven in vehicle-treated, but not thrombin-treated, muscles (Figure 5B). Furthermore, isolectin staining of endothelium three days post-surgery commonly revealed individual collaterals which were much larger than their parallel neighbors in thrombin-treated (4 out of 5 individuals), but not vehicle-treated muscles (Figure 5C & D).

The average diameter of the largest collateral capillaries undergoing arterialization in thrombin-treated muscles was more than double that of vehicle-treated after three days (Figure 5A, E, & F). At this time, most thrombin-treated collaterals had diameters too large to be classified as capillaries yet lacked the complete SMA+ mural cell coverage of mature arterioles. Given another four days to develop, remodeling collaterals in both groups had increased significantly in diameter from the 3-day time point and could now be classified as arterioles due to their diameters and complete SMA+ mural cell coverage. The average diameter of the largest thrombin-treated collaterals was 63% greater than that of the largest vehicle-treated collaterals after seven days (Figure 5A, G, & H).

Thrombin accelerates reperfusion of ischemic regions

Thrombin treatment significantly increased reperfusion of ligated arteriolar watersheds by three days post-ligation compared to vehicle treatment. Average reperfusion was 31% greater in the thrombin-treated group (Figure 6).

Discussion

Our results demonstrate that exogenous thrombin treatment with a dose of 1 NIH unit/gram body weight results in both accelerated formation of new collateral arterioles from collateral capillaries and accelerated tissue reperfusion in the ischemic mouse spinotrapezius. Thrombin-elicited responses in multiple cell types are likely involved in these effects. In uninjured mouse spinotrapezius, thrombin treatment results in the activation of the microvascular endothelium, along with increased leukocyte and mural cell recruitment and/or proliferation.

Exogenous thrombin delivery induces a more persistent upregulation of VCAM-1 than ligation-induced ischemia alone. Upregulation of VCAM-1 in arterioles and venules

adjacent to the ligated arteriolar watershed in muscles receiving ligation alone had subsided by two days post-surgery. In contrast, upregulation of VCAM-1 induced by exogenous thrombin was still present three days post-treatment. Prolonged upregulation of adhesion molecules may increase recruitment of some cell populations in thrombin-treated muscle.

Thrombin-treated non-operated muscles displayed increased infiltration of $CD11b^+$ cells compared to vehicle-treated non-operated muscles. However, this effect was not observed in ligated muscles. This could be due to our small sample size or due to a recruitment plateau for these cells which is attained following ligation-induced ischemia and which the effects of thrombin can not exceed. Regardless, we are unable to conclude that monocyte recruitment induced by exogenous thrombin is additive with monocyte recruitment induced by ischemic injury. The literature suggests that exogenous thrombin should increase monocyte recruitment. Thrombin is known to induce MCP-1 production in endothelial cells[12]. Also, Popovic *et al*. demonstrated that thrombin induced up-regulation of membrane-anchored CX3CL1 in endothelial cells. This resulted in increased monocyte adhesion and the release of MCP-1 from adhered monocytes[45]. Thrombin receptor (PAR-1) activation has been shown to be necessary for MCP-1 dependant leukocyte recruitment *in vivo*[9]. The importance of monocyte recruitment in arteriogenesis is well documented[2, 16, 22, 40, 41].

Thrombin treatment resulted in substantial mast cell infiltration of both non-operated and ligated muscles. Thrombin has been implicated in mast cell activity[49] and induces mast cell degranulation[46]. Mast cells are thought to promote arteriogenesis through cytokine signaling resulting in the recruitment of bone marrow cell populations and the enhancement of vascular cell proliferation and differentiation (for review see [37]). MCP-1 and its receptor, CCR2, play important roles in mast cell migration[11]. In culture, thrombin has been demonstrated to increase MCP-1 production in vascular pericytes[20], endothelial cells[12], and smooth muscle cells[29].

The origin of the SMA+ perivascular mural cells recruited to thrombin-treated capillaries remains unclear. Endothelial-mesenchymal transdifferentiation of endothelial cells, as well as recruitment and/or proliferation of vascular pericytes, smooth muscle cells, and circulating myeloid progenitor cells are all possibilities that remain to be studied in more detail. Collateral capillaries undergoing arterialization often displayed unconnected patches of SMA+ mural cells across their entire lengths on day three post-surgery. Therefore, it does not appear that smooth muscle cells were proliferating and/or migrating in a linear fashion along the endothelium and onto developing collaterals from neighboring arteriole terminals. A subpopulation of endothelial cells has been shown to have the capacity to undergo endothelial-mesenchymal transdifferentiation *in vitro*, becoming smooth muscle-like cells that express SMA and other markers of smooth muscle differentiation[17]. Thrombin and PAR1 have been implicated as participants in this process[1]. Vascular pericytes are smooth muscle-like mural cells involved in vessel stability (for review see [54]). Thrombin has been demonstrated *in vitro* to up-regulate PDGF secretion from endothelial cells[21] and macrophages[52], and PDGF is required for vascular pericyte recruitment[53]. Thrombin is also known to induce smooth muscle cell proliferation[306, 24, 35] and migration[42] *in vitro* while blocking its main receptor, PAR1, has been shown to attenuate smooth muscle cell accumulation in response to vascular injury *in vivo*[28, 51]. Progenitor cells of myeloid origin capable of differentiation into smooth muscle-like cells have been shown to be present in circulating blood[38, 48]. Martin *et al*. demonstrated that these cells can be induced to differentiate into smooth muscle-like cells by thrombin through PAR1 activation[32] *in vitro*. However, the ability of these cells to differentiate into definitive smooth muscle cells *in vivo* remains controversial (see [14] for review). Thrombin-induced effects on mural cells may contribute to the observed accelerated collateral development by providing increased stability to remodeling endothelium. Though we observed SMA+ capillary-sized vessels in

non-operated, thrombin-treated muscles, they lacked substantial diameter increases. It is unlikely that thrombin-induced cell recruitment and proliferation could result in substantial diameter increases in the absence of fluid shear stress changes.

The number of SMA+ intersections did not increase significantly between days three and seven in the thrombin-treated group. Also, examinations of isolectin staining in areas of collateral capillaries undergoing diameter expansion in thrombin-treated muscle after three days often revealed individual remodeling collaterals that were much larger than their parallel neighbor collaterals. Therefore, it does not appear that the difference in numbers of newly formed arteriole collaterals observed at day seven are the result of regression of mature arteriole collaterals in the thrombin-treated group. Rather, these findings imply a thrombin-induced effect on the endothelium of collateral capillaries which results in select capillaries remodeling at a pace greater than that possible in the absence of exogenous thrombin, but at the expense of diameter expansion in parallel collateral capillaries. The reperfusion results demonstrate that this structural remodeling of the microvascular network is beneficial, underscoring that a few large vessels are better than many small vessels for supplying blood flow.

Care must be taken when delivering exogenous thrombin near large vessels. We first attempted to administer a higher dose (1.8 NIH unit/gram body weight) similar to that used in the rabbit hind-limb model by Katsanos *et al*.[18, 27] to undermined spinotrapezius muscles with main vessels exposed beneath, but anesthetized mice stopped breathing almost immediately. We presume this was due to pulmonary embolism, but we did not verify this. Doses of 1NIH unit/gram body weight never had this effect. Because of the inherent risk of thrombin delivery with regards to its role in clotting, more work is needed to determine effective yet safe doses and to examine the use of other downstream effectors of thrombin as the correct combination of downstream effectors (e.g. MCP-1, PDGF, etc.) could mimic thrombin's arteriogenic effects without its inherent dangers.

The applicability of results from studies using young healthy animals is often questionable in regards to treating human diseases which commonly manifest in aged individuals. Westvik *et al.* found that decreased functional recovery in old mice following hind-limb ischemia was due not to impaired angiogenesis, but to impaired arteriogenesis[56]. It is unknown whether the arteriogenic effects of thrombin observed in this study would be applicable in aged individuals, but a treatment to enhance arteriogenesis in these individuals could speed reperfusion following ischemic events and lessen tissue damage.

It is unknown what contribution the induction of small arterioles from capillary collaterals might have to overall tissue reperfusion in peripheral arterial disease. New techniques for examining remodeling of smaller caliber microvessels in hind-limb ischemia models may eventually help address this question. It is logical to speculate that the effects of thrombin on microvasculature remodeling were likely present in the rabbit hind-limb ischemia study[27] and could have contributed in some way to the observed difference in reperfusion following femoral artery excision. The original size of the vessels from which the observed large arteriole collaterals were derived is still unknown. There is no evidence to suggest that small arterioles can not remodel to become high conductance collateral arteries under the right circumstances.

The fact that only one large redundancy in arteriolar flow is sufficient to protect against occlusion is important and is consistent with a computational model prediction previously made by our group[31]. It would not be necessary to induce collaterals in multiple locations to provide protection against ischemic insult. Local delivery of arteriogenic agents to specific points could be effective for inducing collateral development while avoiding systemic

effects of treatment. In particular, local delivery of exogenous thrombin to developing collaterals would be necessary to avoid undesirable side-effects in aged and diseased individuals, as thrombin-induced mitogenic and chemotactic effects could detrimentally contribute to atherosclerosis (for review see[33, 34]) and restenosis[10].

Perspectives

Exogenous thrombin delivery accelerates the formation of collateral arterioles from collateral capillaries in the Balb/c mouse spinotrapezius muscle model of ischemia. This results in accelerated tissue reperfusion following ischemic injury, which may have implications for future treatments of peripheral artery disease and heart disease.

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List of Abbreviations

bFGF	basic fibroblast growth factor
CCR2	chemokine (C-C motif) receptor 2
CX3CL1	chemokine (C-X3-C motif) ligand 1
ICAM-1	intercellular adhesion molecule-1
IL-8	interleukin-8
HIF-1	hypoxia-inducible factor 1
MCP-1	monocyte chemoattractant protein-1
PAR1	protease-activated receptor 1
PDGF	platelet-derived growth factor
SMA	smooth muscle α-actin
TGF-β1	transforming growth factor β-1
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

References

1. Archiniegas E, Neves CY, Candelle D, Cardier JE. Thrombin and its protease-activated receptor-1 (PAR1) participate in the endothelial-mesenchymal transdifferentiation process. DNA Cell Biol. 2004; 23:815–825. [PubMed: 15684708]
2. Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W. Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. J Clin Invest. 1998; 101:40–50. [PubMed: 9421464]
3. Bachhuber BG, Sarembock IJ, Gimple LW, Owens GK. alpha-Thrombin induces transforming growth factor-beta1 mRNA and protein in cultured vascular smooth muscle cells via a proteolytically activated receptor. J Vasc Res. 1997; 34:41–48. [PubMed: 9075824]

4. Bailey AM, O'Neill TJt, Morris CE, Peirce SM. Arteriolar remodeling following ischemic injury extends from capillary to large arteriole in the microcirculation. Microcirculation. 2008; 15:389–404. [PubMed: 18574742]

5. Bar-Shavit R, Kahn AJ, Mann KG, Wilner GD. Identification of a thrombin sequence with growth factor activity on macrophages. Proc Natl Acad Sci U S A. 1986; 83:976–980. [PubMed: 3456576]

6. Bar-Shavit R, Benezra M, Eldor A, Hy-Am E, Fenton JW 2nd, Wilner GD, Vlodavsky I. Thrombin immobilized to extracellular matrix is a potent mitogen for vascular smooth muscle cells: nonenzymatic mode of action. Cell Regul. 1990; 1:453–463. [PubMed: 1963793]

7. Bassus S, Herkert O, Kronemann N, Gorlach A, Bremerich D, Kirchmaier CM, Busse R, Schini-Kerth VB. Thrombin causes vascular endothelial growth factor expression in vascular smooth muscle cells: role of reactive oxygen species. Arterioscler Thromb Vasc Biol. 2001; 21:1550–1555. [PubMed: 11557687]

8. Chalothorn D, Faber JE. Strain-dependent variation in collateral circulatory function in mouse hindlimb. Physiol Genomics. 2010; 42:469–479. [PubMed: 20551146]

9. Chen D, Carpenter A, Abrahams J, Chambers RC, Lechler RI, McVey JH, Dorling A. Protease-activated receptor 1 activation is necessary for monocyte chemoattractant protein 1-dependent leukocyte recruitment in vivo. J Exp Med. 2008; 205:1739–1746. [PubMed: 18606855]

10. Chieng-Yane P, Bocquet A, Letienne R, Bourbon T, Sablayrolles S, Perez M, Hatem SN, Lompre AM, Le Grand B, David-Dufilho M. Protease-activated receptor-1 antagonist F 16618 reduces arterial restenosis by down-regulation of tumor necrosis factor alpha and matrix metalloproteinase 7 expression, migration, and proliferation of vascular smooth muscle cells. J Pharmacol Exp Ther. 2011; 336:643–651. [PubMed: 21139058]

11. Collington SJ, Hallgren J, Pease JE, Jones TG, Rollins BJ, Westwick J, Austen KF, Williams TJ, Gurish MF, Weller CL. The role of the CCL2/CCR2 axis in mouse mast cell migration in vitro and in vivo. J Immunol. 2010; 184:6114–6123. [PubMed: 20427772]

12. Colotta F, Sciacca FL, Sironi M, Luini W, Rabiet MJ, Mantovani A. Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. Am J Pathol. 1994; 144:975–985. [PubMed: 8178946]

13. Cucina A, Borrelli V, Di Carlo A, Pagliei S, Corvino V, Santoro-D'Angelo L, Cavallaro A, Sterpetti AV. Thrombin induces production of growth factors from aortic smooth muscle cells. J Surg Res. 1999; 82:61–66. [PubMed: 10068527]

14. Daniel JM, Sedding DG. Circulating smooth muscle progenitor cells in arterial remodeling. J Mol Cell Cardiol. 2010; 50:273–279. [PubMed: 21047514]

15. Daniel TO, Gibbs VC, Milfay DF, Garovoy MR, Williams LT. Thrombin stimulates c-sis gene expression in microvascular endothelial cells. J Biol Chem. 1986; 261:9579–9582. [PubMed: 2426251]

16. de Groot D, Haverslag RT, Pasterkamp G, de Kleijn DP, Hoefer IE. Targeted deletion of the inhibitory NF-kappaB p50 subunit in bone marrow-derived cells improves collateral growth after arterial occlusion. Cardiovasc Res. 2010; 88:179–185. [PubMed: 20495189]

17. Frid MG, Kale VA, Stenmark KR. Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. Circ Res. 2002; 90:1189–1196. [PubMed: 12065322]

18. Gaffney PJ, Edgell TA. The International and "NIH" units for thrombin--how do they compare? Thromb Haemost. 1995; 74:900–903. [PubMed: 8571318]

19. Gorlach A, Diebold I, Schini-Kerth VB, Berchner-Pfannschmidt U, Roth U, Brandes RP, Kietzmann T, Busse R. Thrombin activates the hypoxia-inducible factor-1 signaling pathway in vascular smooth muscle cells: Role of the p22(phox)-containing NADPH oxidase. Circ Res. 2001; 89:47–54. [PubMed: 11440977]

20. Grandaliano G, Valente AJ, Abboud HE. A novel biologic activity of thrombin: stimulation of monocyte chemotactic protein production. J Exp Med. 1994; 179:1737–1741. [PubMed: 8163952]

21. Grandaliano G, Choudhury GG, Poptic E, Woodruff K, Barnes JL, Abboud HE. Thrombin regulates PDGF expression in bovine glomerular endothelial cells. J Am Soc Nephrol. 1998; 9:583–589. [PubMed: 9555660]

22. Heil M, Ziegelhoeffer T, Wagner S, Fernandez B, Helisch A, Martin S, Tribulova S, Kuziel WA, Bachmann G, Schaper W. Collateral artery growth (arteriogenesis) after experimental arterial occlusion is impaired in mice lacking CC-chemokine receptor-2. Circ Res. 2004; 94:671–677. [PubMed: 14963007]

23. Herbert JM, Dupuy E, Laplace MC, Zini JM, Bar Shavit R, Tobelem G. Thrombin induces endothelial cell growth via both a proteolytic and a non-proteolytic pathway. Biochem J. 1994; 303(Pt 1):227–231. [PubMed: 7945245]

24. Kanthou C, Parry G, Wijelath E, Kakkar VV, Demoliou-Mason C. Thrombin-induced proliferation and expression of platelet-derived growth factor-A chain gene in human vascular smooth muscle cells. FEBS Lett. 1992; 314:143–148. [PubMed: 1333990]

25. Kaplanski G, Fabrigoule M, Boulay V, Dinarello CA, Bongrand P, Kaplanski S, Farnarier C. Thrombin induces endothelial type II activation in vitro: IL-1 and TNF-alpha-independent IL-8 secretion and E-selectin expression. J Immunol. 1997; 158:5435–5441. [PubMed: 9164965]

26. Kaplanski G, Marin V, Fabrigoule M, Boulay V, Benoliel AM, Bongrand P, Kaplanski S, Farnarier C. Thrombin-activated human endothelial cells support monocyte adhesion in vitro following expression of intercellular adhesion molecule-1 (ICAM-1; CD54) and vascular cell adhesion molecule-1 (VCAM-1; CD106). Blood. 1998; 92:1259–1267. [PubMed: 9694714]

27. Katsanos K, Karnabatidis D, Diamantopoulos A, Kagadis GC, Ravazoula P, Nikiforidis GC, Siablis D, Tsopanoglou NE. Thrombin promotes arteriogenesis and hemodynamic recovery in a rabbit hindlimb ischemia model. J Vasc Surg. 2009; 49:1000–1012. [PubMed: 19217750]

28. Kogushi M, Matsuoka T, Kuramochi H, Murakami K, Kawata T, Kimura A, Chiba K, Musha T, Suzuki S, Kawahara T, Kajiwara A, Hishinuma I. Oral administration of the thrombin receptor antagonist E5555 (atopaxar) attenuates intimal thickening following balloon injury in rats. Eur J Pharmacol. 2011; 666:158–164. [PubMed: 21635884]

29. Kranzhofer R, Clinton SK, Ishii K, Coughlin SR, Fenton JW 2nd, Libby P. Thrombin potently stimulates cytokine production in human vascular smooth muscle cells but not in mononuclear phagocytes. Circ Res. 1996; 79:286–294. [PubMed: 8756006]

30. Lin CC, Shyr MH, Chien CS, Wang CC, Chiu CT, Hsiao LD, Yang CM. Thrombin-stimulated cell proliferation mediated through activation of Ras/Raf/MEK/MAPK pathway in canine cultured tracheal smooth muscle cells. Cell Signal. 2002; 14:265–275. [PubMed: 11812655]

31. Mac Gabhann F, Peirce SM. Collateral capillary arterialization following arteriolar ligation in murine skeletal muscle. Microcirculation. 2010; 17:333–347. [PubMed: 20618691]

32. Martin K, Weiss S, Metharom P, Schmeckpeper J, Hynes B, O'Sullivan J, Caplice N. Thrombin stimulates smooth muscle cell differentiation from peripheral blood mononuclear cells via protease-activated receptor-1, RhoA, and myocardin. Circ Res. 2009; 105:214–218. [PubMed: 19574550]

33. Martorell L, Martinez-Gonzalez J, Rodriguez C, Gentile M, Calvayrac O, Badimon L. Thrombin and protease-activated receptors (PARs) in atherothrombosis. Thromb Haemost. 2008; 99:305–315. [PubMed: 18278179]

34. Marutsuka K, Hatakeyama K, Yamashita A, Asada Y. Role of thrombogenic factors in the development of atherosclerosis. J Atheroscler Thromb. 2005; 12:1–8. [PubMed: 15725689]

35. McNamara CA, Sarembock IJ, Gimple LW, Fenton JW 2nd, Coughlin SR, Owens GK. Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. J Clin Invest. 1993; 91:94–98. [PubMed: 8380817]

36. 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. 2007; 116:975–983. [PubMed: 17679611]

37. Meisner JK, Price RJ. Spatial and temporal coordination of bone marrow-derived cell activity during arteriogenesis: regulation of the endogenous response and therapeutic implications. Microcirculation. 2010; 17:583–599. [PubMed: 21044213]

38. Metharom P, Liu C, Wang S, Stalboerger P, Chen G, Doyle B, Ikeda Y, Caplice NM. Myeloid lineage of high proliferative potential human smooth muscle outgrowth cells circulating in blood

and vasculogenic smooth muscle-like cells in vivo. Atherosclerosis. 2008; 198:29–38. [PubMed: 17963771]

39. Naldini A, Carney DH, Bocci V, Klimpel KD, Asuncion M, Soares LE, Klimpel GR. Thrombin enhances T cell proliferative responses and cytokine production. Cell Immunol. 1993; 147:367–377. [PubMed: 8453678]
40. Nickerson MM, Burke CW, Meisner JK, Shuptrine CW, Song J, Price RJ. Capillary arterialization requires the bone-marrow-derived cell (BMC)-specific expression of chemokine (C-C motif) receptor-2, but BMCs do not transdifferentiate into microvascular smooth muscle. Angiogenesis. 2009; 12:355–363. [PubMed: 19777360]
41. Nickerson MM, Song J, Meisner JK, Bajikar S, Burke CW, Shuptrine CW, Owens GK, Skalak TC, Price RJ. Bone marrow-derived cell-specific chemokine (C-C motif) receptor-2 expression is required for arteriolar remodeling. Arterioscler Thromb Vasc Biol. 2009; 29:1794–1801. [PubMed: 19734197]
42. Noda-Heiny H, Sobel BE. Vascular smooth muscle cell migration mediated by thrombin and urokinase receptor. Am J Physiol. 1995; 268:C1195–C1201. [PubMed: 7762612]
43. Peirce SM, Skalak TC. Microvascular remodeling: a complex continuum spanning angiogenesis to arteriogenesis. Microcirculation. 2003; 10:99–111. [PubMed: 12610666]
44. Petaja J. Inflammation and coagulation. An overview. Thromb Res. 2011; 127 Suppl 2:S34–S37. [PubMed: 21193113]
45. Popovic M, Laumonnier Y, Burysek L, Syrovets T, Simmet T. Thrombin-induced expression of endothelial CX3CL1 potentiates monocyte CCL2 production and transendothelial migration. J Leukoc Biol. 2008; 84:215–223. [PubMed: 18436581]
46. Razin E, Marx G. Thrombin-induced degranulation of cultured bone marrow-derived mast cells. J Immunol. 1984; 133:3282–3285. [PubMed: 6208276]
47. Schaper W. Collateral circulation: past and present. Basic Res Cardiol. 2009; 104:5–21. [PubMed: 19101749]
48. Simper D, Stalboerger PG, Panetta CJ, Wang S, Caplice NM. Smooth muscle progenitor cells in human blood. Circulation. 2002; 106:1199–1204. [PubMed: 12208793]
49. Strukova SM, Chistov IV, Umarova BA, Dugina TN, Storozhevykh TP, Pinelis VG, Glusa E. Modulation of mast cell activity by a peptide agonist of the thrombin receptor: role of nitric oxide. Biochemistry (Mosc). 1999; 64:658–664. [PubMed: 10395981]
50. Sugama Y, Tiruppathi C, offakidevi K, Andersen TT, Fenton JW 2nd, Malik AB. Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion. J Cell Biol. 1992; 119:935–944. [PubMed: 1385447]
51. Takada M, Tanaka H, Yamada T, Ito O, Kogushi M, Yanagimachi M, Kawamura T, Musha T, Yoshida F, Ito M, Kobayashi H, Yoshitake S, Saito I. Antibody to thrombin receptor inhibits neointimal smooth muscle cell accumulation without causing inhibition of platelet aggregation or altering hemostatic parameters after angioplasty in rat. Circ Res. 1998; 82:980–987. [PubMed: 9598595]
52. Tani K, Ogushi F, Takahashi H, Kawano T, Endo T, Sone S. Thrombin stimulates platelet-derived growth factor release by alveolar macrophages in rats--significance in bleomycin-induced pulmonary fibrosis. J Med Invest. 1997; 44:59–65. [PubMed: 9395719]
53. Tengood JE, Kovach KM, Vescovi PE, Russell AJ, Little SR. Sequential delivery of vascular endothelial growth factor and sphingosine 1-phosphate for angiogenesis. Biomaterials. 2010; 31:7805–7812. [PubMed: 20674008]
54. von Tell D, Armulik A, Betsholtz C. Pericytes and vascular stability. Exp Cell Res. 2006; 312:623–629. [PubMed: 16303125]
55. Wenzel UO, Fouqueray B, Grandaliano G, Kim YS, Karamitsos C, Valente AJ, Abboud HE. Thrombin regulates expression of monocyte chemoattractant protein-1 in vascular smooth muscle cells. Circ Res. 1995; 77:503–509. [PubMed: 7641321]
56. Westvik TS, Fitzgerald TN, Muto A, Maloney SP, Pimiento JM, Fancher TT, Magri D, Westvik HH, Nishibe T, Velazquez OC, Dardik A. Limb ischemia after iliac ligation in aged mice stimulates angiogenesis without arteriogenesis. J Vasc Surg. 2009; 49:464–473. [PubMed: 19028053]

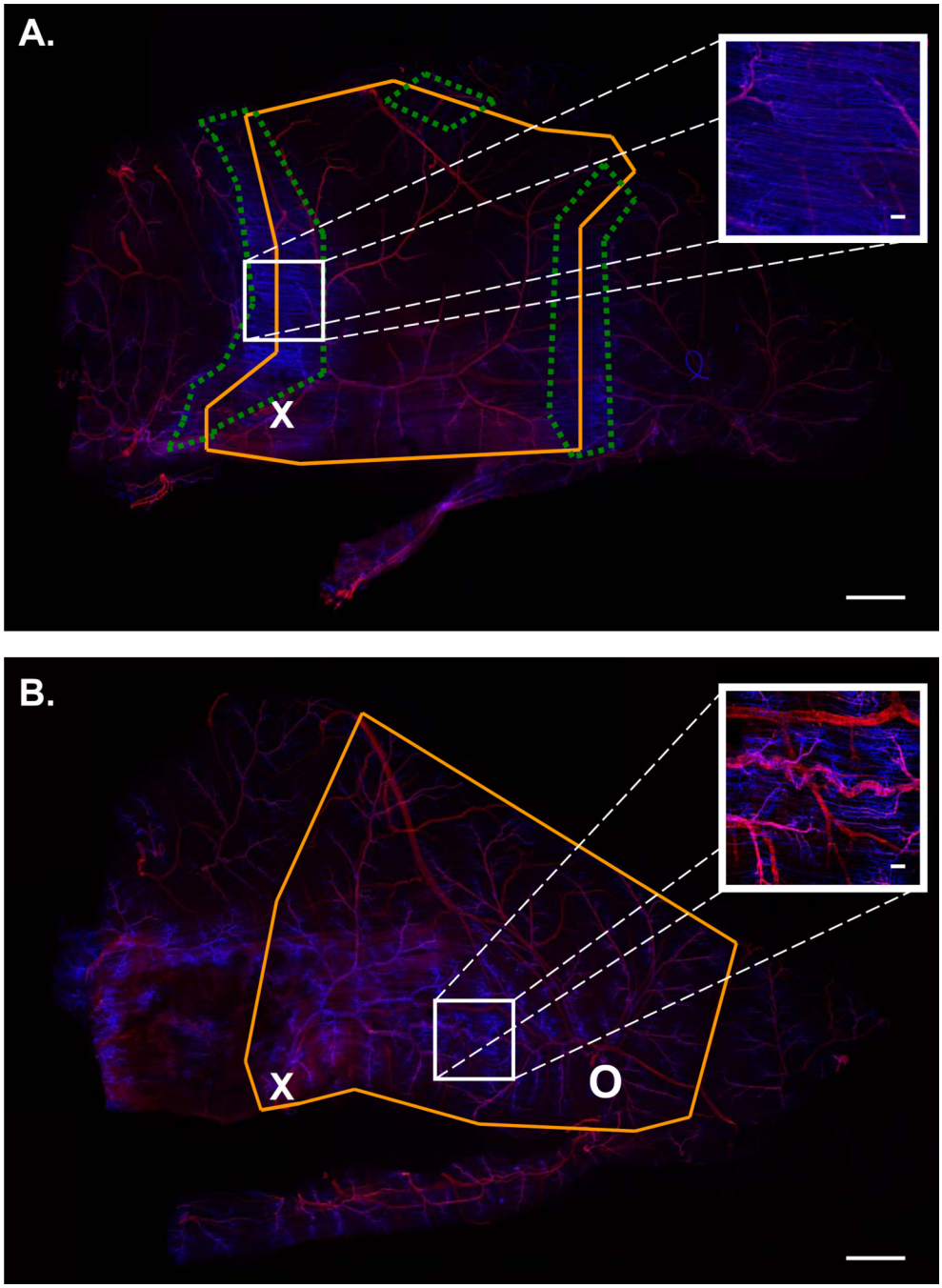


Figure 1. Pre-existing arteriole collaterals prevent collateral capillary arterialization

A. 40X Balb/c spinotrapezius three days post surgery exhibiting remodeling of collateral capillaries. **B.** 40X Balb/c spinotrapezius exhibiting a single pre-existing arteriole collateral in the ligated arteriolar watershed. Note lack of remodeling collateral capillaries. X denotes the entry point of the ligated feeder arteriole. O denotes the entry point of a redundant feeding arteriole. The orange solid line denotes the boundaries of the ligated arteriolar watershed. Green dotted lines denote areas of collateral capillaries undergoing diameter expansion. The insets show remodeling at 100X. Red staining is smooth muscle α-actin and blue staining is isolectin. Scales bars = 500 μm and 50 μm (inset).

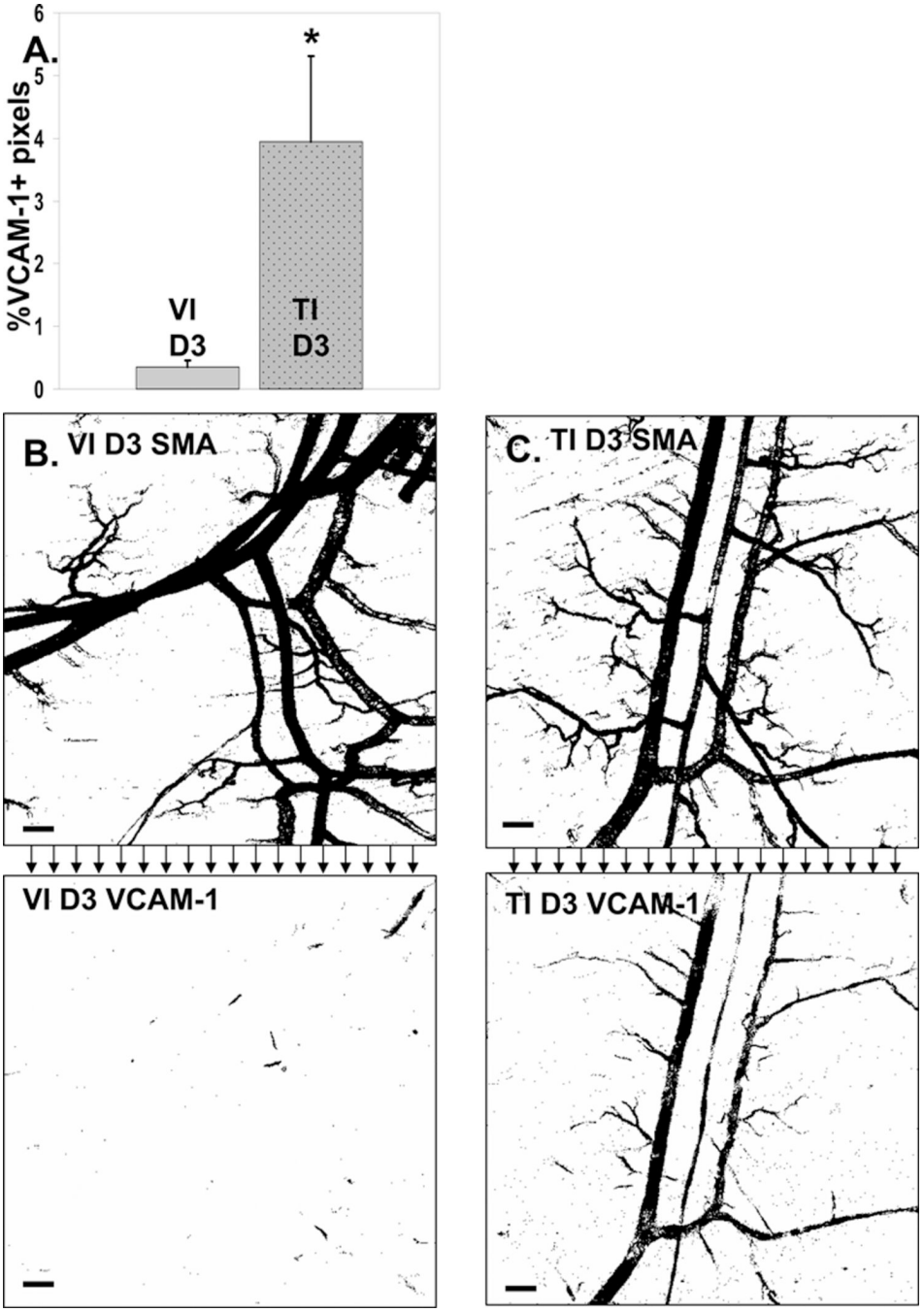
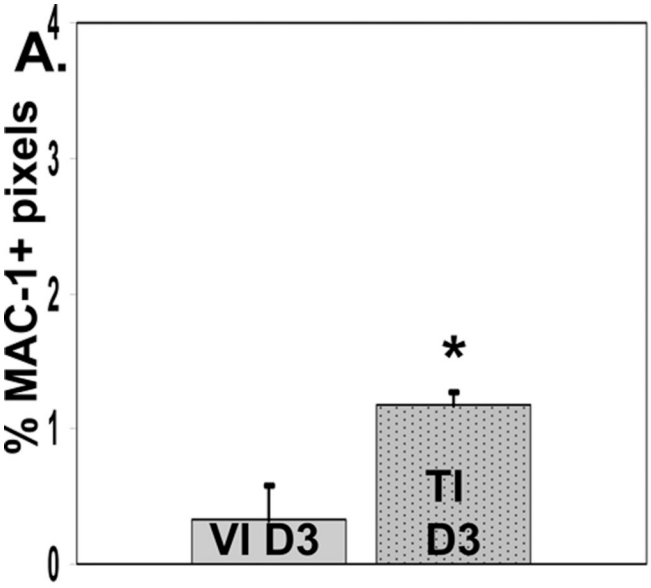
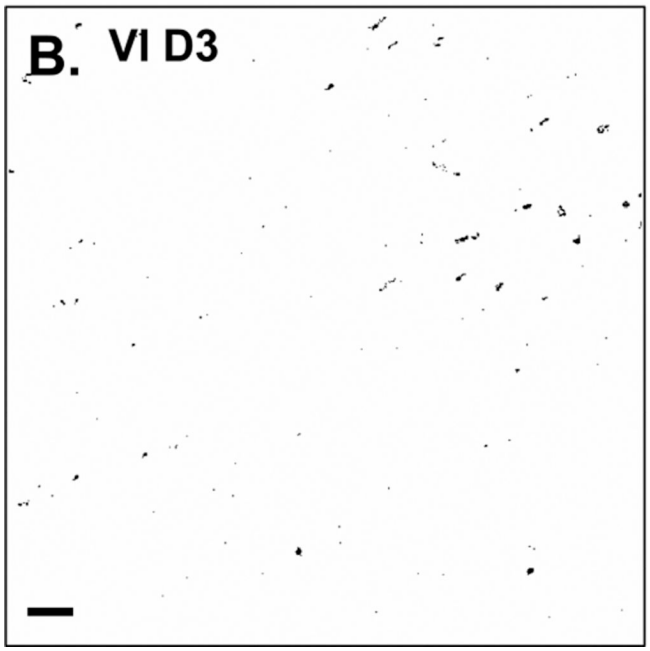
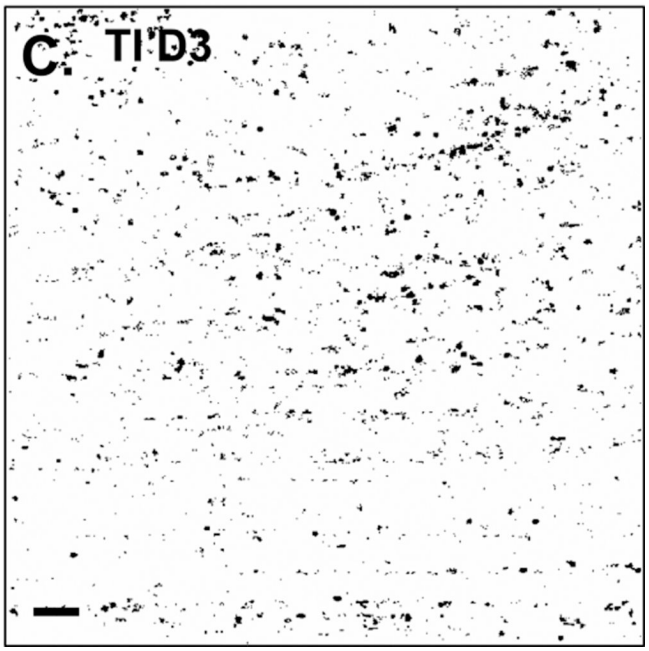


Figure 2. Thrombin activates the microvascular endothelium

A. Thrombin injection (TI, N=4) increases VCAM-1 expression in non-operated arterioles and venules compared to vehicle injection (VI, N=4) as measured by positive pixels in 100X fields of view after three days (D3). * denotes $p<0.05$. **B.** Smooth muscle α-actin (SMA) and VCAM-1 expression of a vehicle-injected arteriole/venule pair after three days. **C**. SMA and VCAM-1 expression of a thrombin-injected arteriole/venule pair after three days. VCAM-1 expression is markedly upregulated. Error bars = standard error. Scales bars = 50 μm.







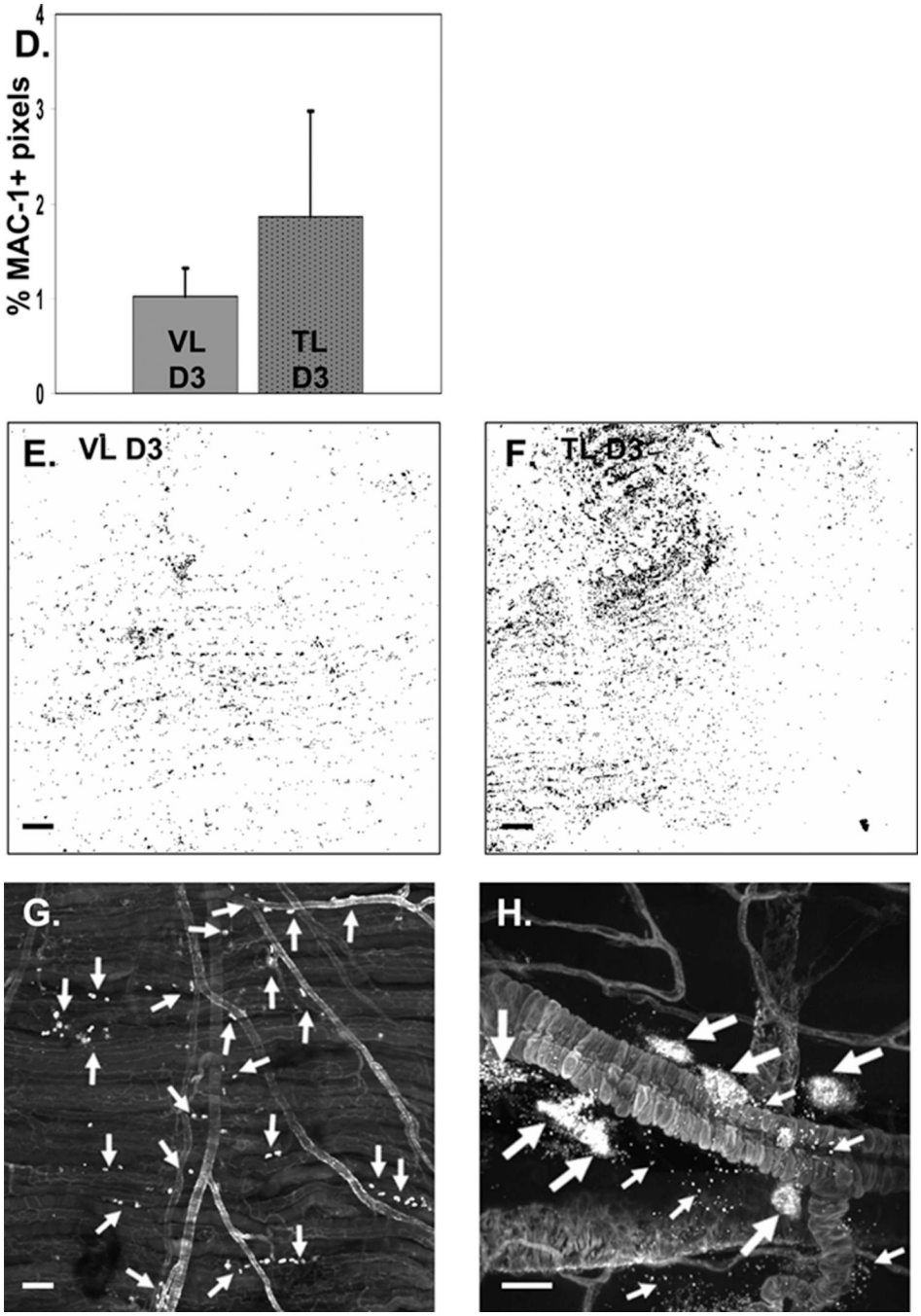
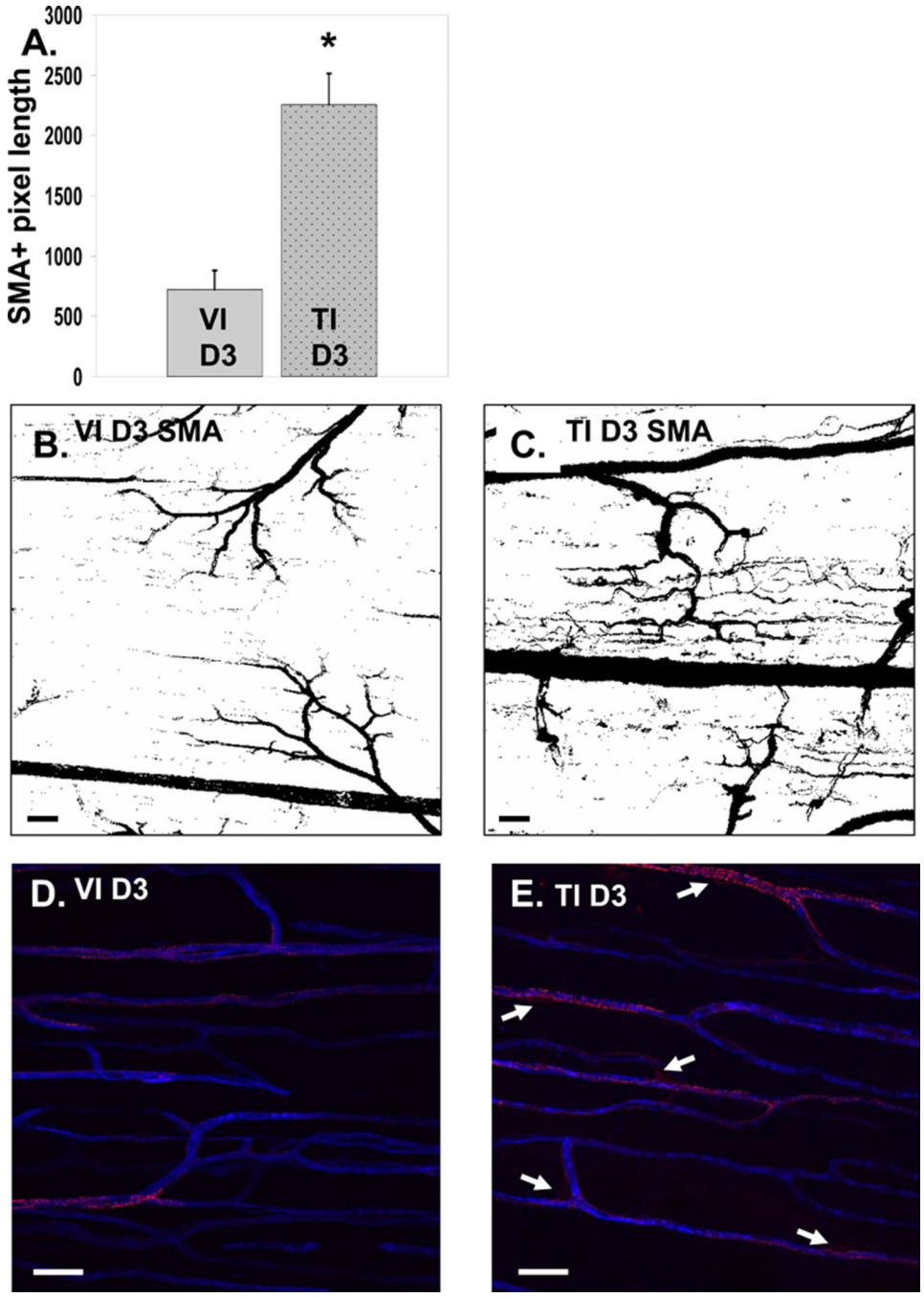


Figure 3. Thrombin promotes leukocyte infiltration

A. Thrombin injection (TI, N=4) in non-operated muscles significantly increases CD11b+ leukocyte infiltration compared to vehicle injection (VI, N=4) as measured by positive pixels in 100X fields of view after three days (D3). * denotes $p<0.05$. **B.** CD11b staining in VI D3 (100X field, caudal spinotrapezius). **C.** CD11b staining in TI D3 (100X field, caudal spinotrapezius displaying mural cell recruitment to capillary-sized vessels). **D.** CD11b+ leukocyte infiltration in 100X fields containing microvascular remodeling was not significantly different between vehicle-treated ligated (VL, N=5) and thrombin-treated ligated (TL, N=5) muscles after three days (D3). p=0.48. **E.** CD11b staining in VL D3 (100X field containing remoldeling collaterals). **F.** CD11b staining in TL D3 (100X field

containing remoldeling collaterals). **G.** 100X image displaying mast cell infiltration induced by thrombin treatment. Arrows indicate mast cells. **H.** 600X image of mast cells degranulating adjacent to an arteriole in thrombin-treated muscle. Large arrows indicate mast cells. Small arrows indicate released granules. Error bars = standard error. Scale bars (B, C, E, F, G) = 50 μm, (H) = 20 μm.



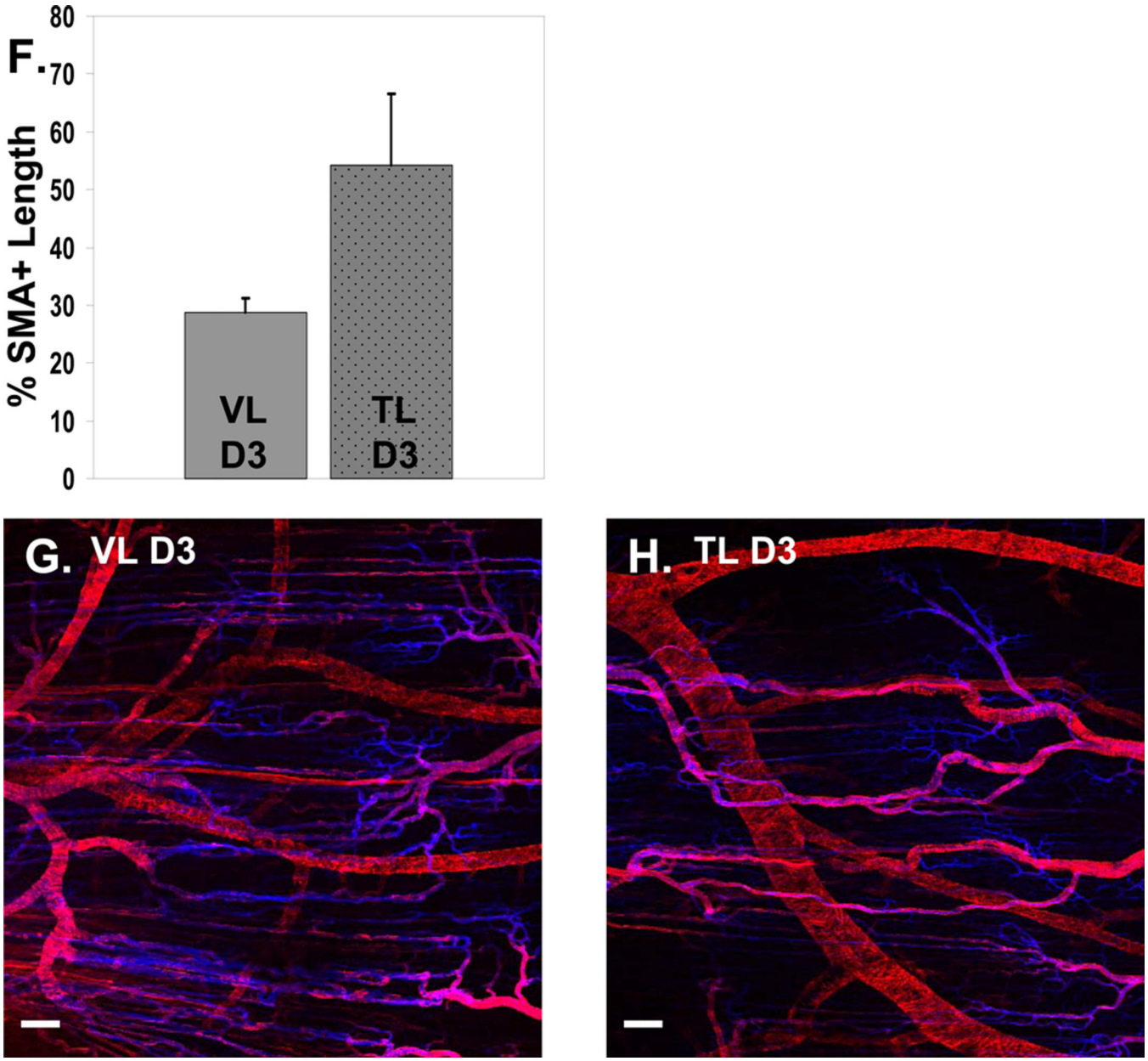
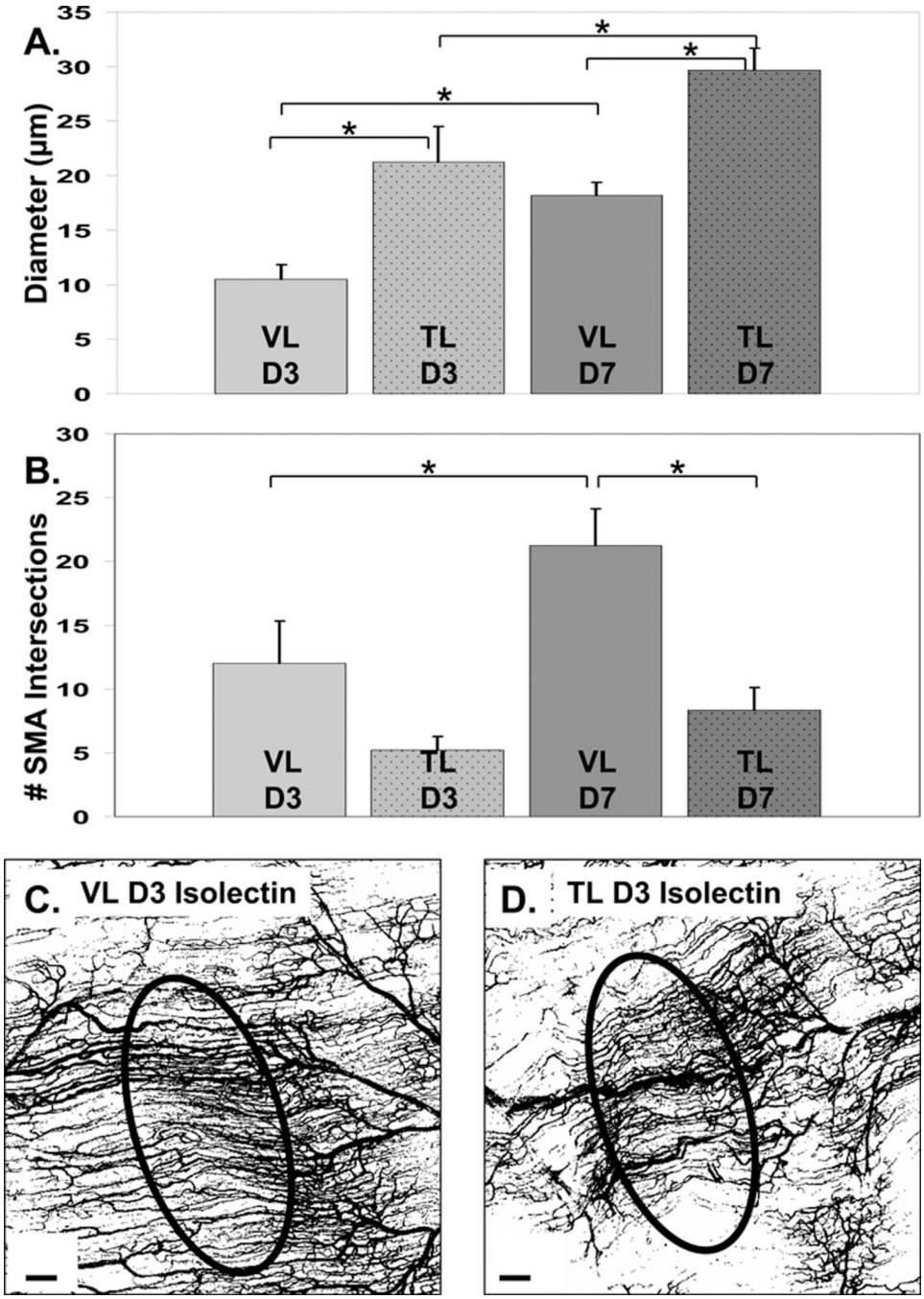


Figure 4. Thrombin promotes SMA+ mural cell investment

A. Thrombin injection (TI, N=4) in non-operated muscles increases smooth muscle α-actin (SMA)+ pixel length on vessels 10 μm and less in diameter compared to vehicle injection (VI, N=4) in 100X fields of view after three days (D3). * denotes $p<0.01$. **B.** Capillary-sized vessels at arteriole terminals in VI D3 posterior spinotrapezius lack investment of SMA+ mural cells. **C.** Capillary-sized vessels at arteriole terminals in TI D3 posterior spinotrapezius display investment of SMA+ mural cells. **D.** VI D3 do not display SMA+ cell bodies in contact with the endothelium at borders between SMA+ and SMA− vessels in 600X fields. **E.** TI D3 display SMA+ cell bodies (arrows indicate locations) in contact with the endothelium at borders between SMA+ and SMA− vessels in 600X fields. **F.** SMA+ mural cell investment tended to be greater on the largest remodeling collaterals in thrombin-treated ligated (TL, N=5) versus vehicle-treated ligated (VL, N=5) three days (D3) post-ligation. $p = 0.08$. **G.** Example VL D3 displaying average SMA+ (red) mural cell coverage.

H. Example TL D3 displaying increased SMA+ (red) mural cell coverage. Blue staining is isolectin. Error bars = standard error. Scale bars (B, C, G, H) = 50 μm, (D, E) = 20 μm.



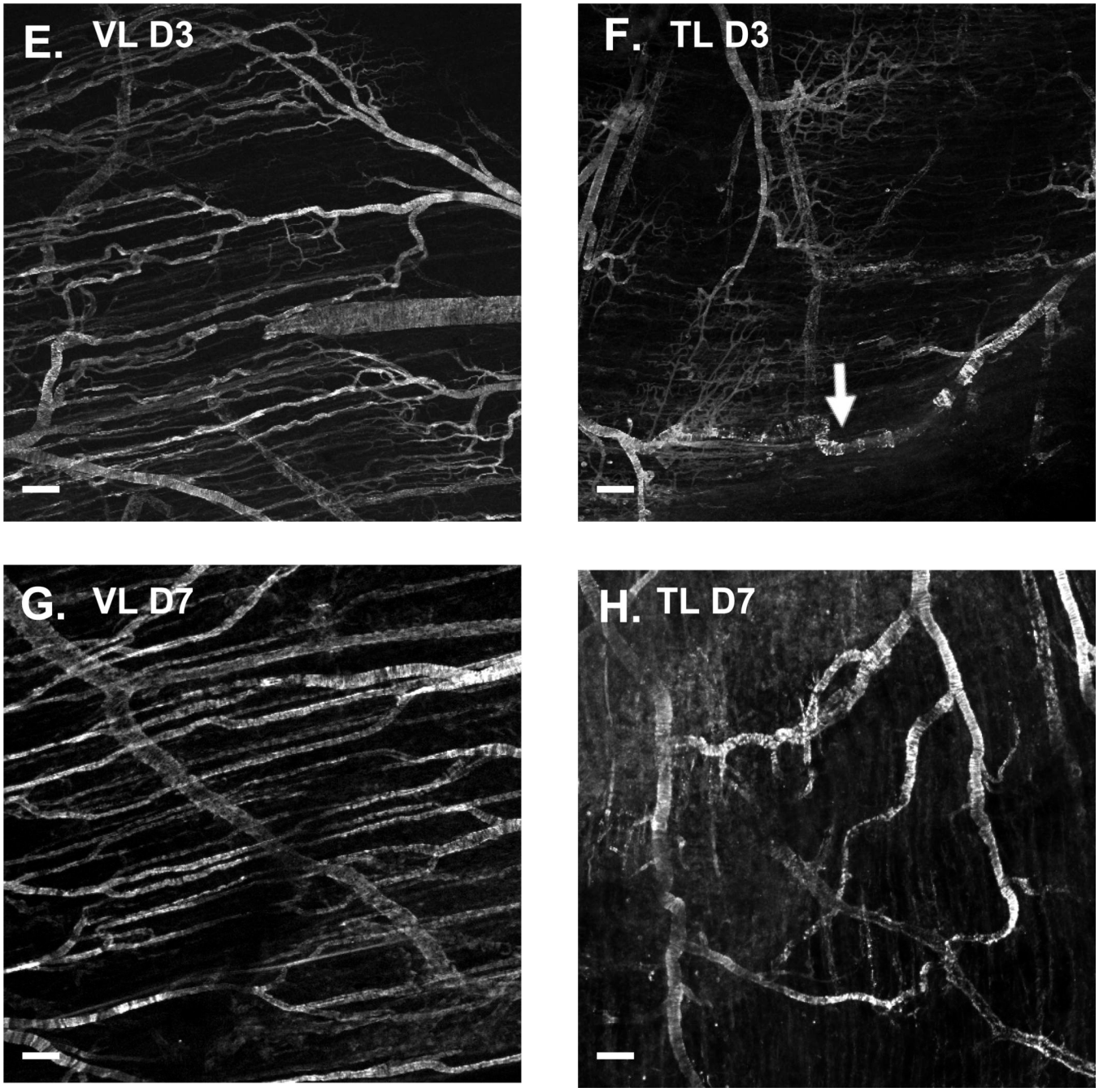


Figure 5. Thrombin accelerates collateral capillary arterialization in the presence of ischemia

A. Thrombin treatment significantly increased maximum collateral diameters achieved after both three and seven days. Maximum diameters achieved increased significantly with time in both treatment groups. VL = vehicle-treated ligated, TL = thrombin-treated ligated, D3 = three days, D7 = seven days. VL D3 N=5, TL D3 N=5, VL D7 N=4, TL D7 N=3. * denotes $p<0.05$. **B.** Smooth muscle α-actin (SMA) + intersections increase with time in VL but not TL in 100X fields containing the largest collateral per muscle. The number of parallel SMA + collaterals in fields containing the largest collateral per muscle is significantly less in TL D7 than VL D7. * denotes $p<0.05$. **C.** Isolectin staining of VL D3 endothelium displaying typical numerous parallel collaterals of similar diameter. **D.** Isolectin staining of TL D3

endothelium displaying typical selective enlargement of few parallel collaterals. **E, F, G, H.** Representative remodeling collaterals from each treatment and time point. Note the increased diameters and decreased number of remodeling parallel collaterals in TL versus VL. The arrow in F indicates the remodeling collateral. Error bars = standard error. Scale bars = 50 μm.

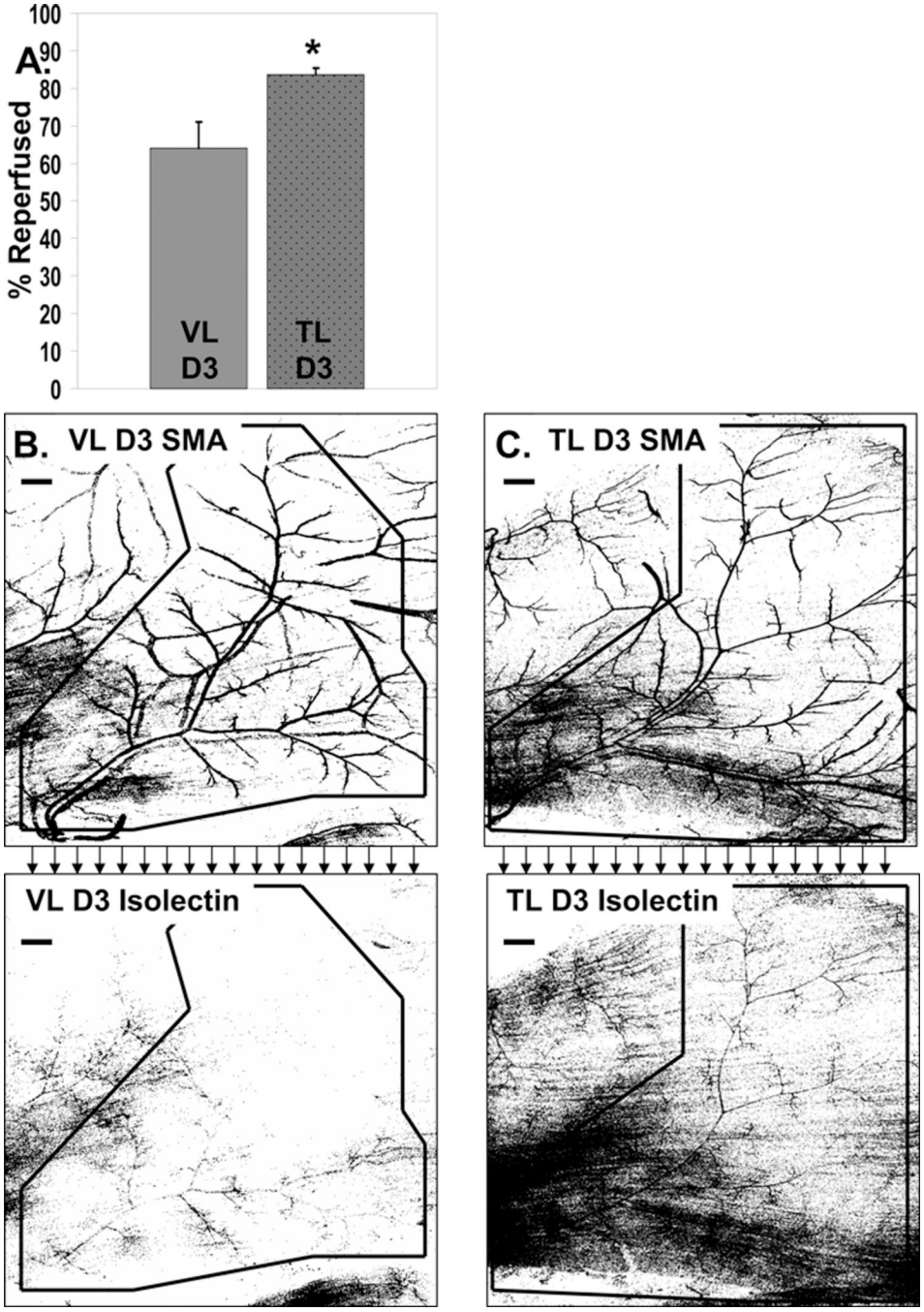


Figure 6. Thrombin accelerates reperfusion of ischemic regions

A. Thrombin treatment (TL, N=5) significantly increased reperfusion of the ligated arteriolar watershed after three days (D3) compared to vehicle treatment (VL, N=5). * denotes $p<0.05$. B. Smooth muscle α-actin (SMA) and isolectin staining showing reperfusion in VL D3. The black lines denote the boundary of the ligated arteriolar watershed. **C.** SMA and isolectin staining showing increased reperfusion in TL D3. The black lines denote the boundary of the ligated arteriolar watershed. Error bars = standard error. Scale bars = 50 μm.