Ultrasonic Microbubble Destruction Stimulates Therapeutic Arteriogenesis via the CD18-Dependent Recruitment of Bone Marrow–Derived Cells

John C. Chappell, Ji Song, Alexander L. Klibanov, Richard J. Price

Objective—We have previously shown that, under certain conditions, ultrasonic microbubble destruction creates arteriogenesis and angiogenesis in skeletal muscle. Here, we tested whether this neovascularization response enhances hyperemia in a rat model of arterial insufficiency and is dependent on the recruitment of bone marrow–derived cells (BMDCs) to treated tissues via a β2 integrin (CD18)-dependent mechanism.

Methods and Results—Sprague-Dawley rats, C57BL/6 wild-type mice, and C57BL/6 chimeric mice engrafted with BMDCs from either GFP⁺ or CD18⁻⁻ mice received bilateral femoral artery ligations. Microbubbles (MBs) were intravenously injected, and one gracilis muscle was exposed to pulsed 1 MHz ultrasound (US). Rat hindlimbs exhibited significant increases in adenosine-induced hyperemia and arteriogenesis compared to contralateral controls at 14 and 28 days posttreatment. US-MB–treated wild-type C57BL/6 mice exhibited significant arteriogenesis, angiogenesis, and CD11b⁺ monocyte recruitment; however, these responses were all completely blocked in CD18⁻⁻ chimeric mice. The number of BMDCs increased in US-MB–treated muscles of GFP⁺ chimeric mice; however, GFP⁺ BMDCs did not incorporate into microvessels as vascular cells.

Conclusion—in skeletal muscle affected by arterial occlusion, arteriogenesis and hyperemia can be significantly enhanced by ultrasonic MB destruction. This response depends on the recruitment of US-MB treatment, but not vascular incorporation, of BMDCs via a CD18-dependent mechanism. (Arterioscler Thromb Vasc Biol. 2008;28:000-000)

Key Words: ultrasonics ■ contrast media ■ arteriogenesis ■ microcirculation ■ marrow-derived cells

Oclusion of the major arteries as a result of atherosclerosis or diabetes often leads to angiogenesis, and the formation of skin ulcers. Although surgical interventions are usually successful, many patients are not amenable to these procedures. For this reason, there is a need to develop new strategies for revascularizing ischemic tissues with minimal surgical invasiveness. To date, promising therapeutic neovascularization methods have been based on the injection or delivery of cytokines, growth factors, growth factor genes, or proangiogenic cells. Although specifically designed to enhance the contrast in diagnostic ultrasound (US) images, microbubbles (MBs) have been used as potential tools in therapeutic strategies. In particular, the use of MBs for targeted drug and gene delivery to large arteries and tissue has received considerable attention. We have recently explored the use of contrast agent MBs in conjunction with US to stimulate neovascularization. Our studies indicate that, when US-MB interactions are tailored to create capillary poration in skeletal muscle, vascular remodeling and enhanced hyperemia may ensue. Furthermore, this treatment enhances hyperemia in skeletal muscle, particularly in regions of low blood flow, through the formation and lumenal expansion of arterioles spanning low and normal perfusion regions. Moreover, we have confirmed that US-MB interactions elicit angiogenic and arteriogenic responses in normal mouse skeletal muscle. These results from the rat and mouse motivate the current study in which we test hypotheses. First, using modifications of our previous US-MB treatment protocol in the rat hindlimb model of arterial insufficiency, we tested the hypothesis that ultrasonic MB destruction augments not only arteriogenesis, but also total perfusion in regions of limited hyperemia within the gracilis adductor muscle. Second, we tested the hypothesis that arteriogenesis in response to the US-MB treatment requires the recruitment of bone marrow–derived cells (BMDCs) through the use of chimeric mice in which BMDCs lack CD18 expression. CD18 is the β2 integrin subunit, mediating BMDC firm adhesion to venules during inflammation. Finally, we tested the hypothesis that a short-term (ie, within 24 hours) reduction in perfusion, a therapeutic detriment and possibly an auxiliary long-term (ie, 14 to 28 days) stimulus for neovascularization, is created by US-MB treatment.

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Methods

Animal Groups and Hindlimb Models of Arterial Insufficiency

Animal studies were approved by the Animal Care and Use Committee at the University of Virginia and conformed to the American Heart Association Guidelines for the Use of Animals in Research. The Table summarizes the number of animals "n", the experimental procedures, and the timelines for each group. The chimeric mice in Groups V through IX were generated by engrafting host C57BL/6J mice with bone marrow from C57BL/6J, CD18−/−, CD18−/−/GFP donor mice as described in the Online Supplement.

Microvascular Remodeling and Bone Marrow–Derived Cell Recruitment

At the time of tissue harvest, animals from Groups II through IX were reanesthetized. Gracilis muscles were exposed, vasodilated by superfusion with 10−4 mol/L adenosine in Ringer’s solution, perfusion-fixed from an aortic cannula with a pressure of 100 mm Hg, and then dissected free. Whole muscles were incubated in 1,200 Cy3-conjugated monoclonal antismooth muscle (SM)-actin overnight (clone 1A4; Sigma), washed, and imaged as whole mounts using confocal microscopy. Whole muscle montages were reconstructed from individual confocal fields-of-view and analyzed to generate arteriolar area density, arteriolar length density, arteriolar and venular diameters, or arteriolar-line intersection metrics as described in the Online Supplement.

Ultrasonic-Microbubble Treatment

The gracilis adductor muscle of 1 hindlimb was exposed to ultrasonic MB destruction 14 and 3 days after AO for rats and mice, respectively, as shown in supplemental Figure 1 using previously described methods. A shorter experimental duration was used for Groups V through IX because preliminary work had shown robust arteriogenesis in the mouse with this protocol. Therefore, we identified a potential mechanistic link between CD18 and arteriogenesis in the mouse with this protocol. Therefore, we identified a potential mechanistic link between CD18 and arteriogenesis in the mouse with this protocol. Therefore, we identified a potential mechanistic link between CD18 and arteriogenesis in the mouse with this protocol.

Ultrasonic-Microbubble Treatment Enhances Arteriogenesis and Flow Capacity in a Rat Hindlimb Model of Arterial Insufficiency

Figure 1A through 1D depict confocal montages of SM α-actin-labeled Group II and III whole mount gracilis muscles. The ratio of SM α-actin−/− vessel area to total en face muscle area in AO+US+MB muscles was 38% and 31% greater than AO+MB controls at 14 and 28 days posttreatment, respectively (Figure 1E). Vessel intersection analysis of AO+US+MB muscles (Figure 1F) at 14 and 28 days after treatment revealed significant increases of, respectively, 53%
and 32% over AO+MB controls. Note that the results from Figure 1E and 1F are derived only from vessels >35 µm in diameter. Presellected first and second order arterioles (A1 and A2) in the AO+US+MB group exhibited maximum diameters that were significantly greater than the diameters of corresponding arterioles in AO+MB controls (Figure 1G). AO+US+MB muscles exhibited significant 39% and 27% increases in adenosine-induced hyperemia when compared to MB muscles exhibited significant 39% and 27% increases in adenosine-induced hyperemia when compared to MB muscles of WT mice does not occur in CD18−/− chimeric mice. Significant increases in arterial vessel length density and in arterial vessel-line intersections per muscle were observed for AO+US+MB muscles from WT, WT chimeric, and GFP+ chimeric mice as compared to AO+MB controls (Figure 2E and 2F). However, in CD18−/− chimeric mice, the US-MB treatment elicited no detectable changes in these arteriogenesis metrics. Figure 3A through 3D show SM α-actin+ microvessel profiles from AO+US+MB and AO+MB muscle sections of WT and CD18−/− chimeric mice. Consistent with the whole-mount data (Figure 2), SM α-actin+ microvessel profiles, per muscle fiber was significantly increased in AO+US+MB muscles from WT, WT chimeric, and GFP+ chimeric mice but was unchanged in AO+US+MB treated muscles from CD18−/− chimeric mice (Figure 3E). Grouping these data by diameter revealed that significant increases were only observed in the <10 µm arterioles (Figure III in the Online Supplement). Supplemental Figure IVA through IVD illustrate BS-1 lectin+ microvessel profiles from WT and CD18−/− chimeric mice. Here, the number of capillaries per muscle fiber increased with treatment (AO+US+MB) for WT, WT chimeric, and GFP+ chimeric mice in comparison to AO+MB controls; however, CD18−/− chimeras showed no differences between treatment groups (Figure IVE).

Figure 1. Ultrasound MB destruction stimulates arteriogenesis and enhanced flow. A–D, Confocal images of SM α-actin labeled AO+US+MB (B, D) and AO+MB (A, C) gracilis muscles. Bar represents 1 mm. E–H, Bar graphs of SM α-actin+ vessel area density in AO (A1), AO+MB control muscles at 14 and 28 days, and A2) in the AO+US+MB muscle sections from WT, WT chimeric, and GFP+ chimeric mice as compared to AO+MB controls (Figure 2E and 2F). However, in CD18−/− chimeric mice, the US-MB treatment elicited no detectable changes in these arteriogenesis metrics. Grouping these data by diameter revealed that significant increases were only observed in the <10 µm arterioles (Figure III in the Online Supplement). Supplemental Figure IVA through IVD illustrate BS-1 lectin+ microvessel profiles from WT and CD18−/− chimeric mice. Here, the number of capillaries per muscle fiber increased with treatment (AO+US+MB) for WT, WT chimeric, and GFP+ chimeric mice in comparison to AO+MB controls; however, CD18−/− chimeras showed no differences between treatment groups (Figure IVE).

Bone Marrow–Derived Cells Are Recruited to Ultrasound Microbubble–Treated Muscles Through CD18 But Do Not Incorporate Into Growing Microvessels

Immunostaining for CD18 in AO+US+MB–treated muscle sections from WT chimeric and CD18−/− chimeric mice was used to verify the effectiveness of the bone marrow engraftment (Online Supplement, Figure V). CD11b-stained muscle sections from AO+US+MB– and AO+MB–treated WT and CD18−/− chimeric mice are shown in Figure 4A through 4D. For WT mice, a 2-fold increase in CD11b+ cells (monocytes) per muscle fiber was observed for AO+US+MB muscles as compared to AO+MB controls (Figure 4E). For WT chimeric mice, the apparent 39% increase in CD11b+ cells per muscle...
fiber between AO+US+MB and AO+MB was not significant (probability value=0.25). We observed no difference in CD11b+ cell density between AO+US+MB and AO+MB muscles from CD18−/− chimeric mice.

GFP+ BMDCs were also observed in muscle sections. AO+US+MB muscles showed a significant 2-fold increase in GFP+ cells per fiber over AO+MB controls (supplemental Figure VI). We also scrutinized these sections for the potential incorporation of GFP+ cells into microvessels. We observed ~4000 vessel profiles but found no evidence for the transdifferentiation and/or incorporation of GFP+ BMDCs into vascular cells or structures (supplemental Figure VII).

**Discussion**

Ultrasonic MB destruction enhances vascular remodeling in normal17,18 and ischemic skeletal muscle19; however, the molecular and cellular mechanisms underlying this phenomenon remain unknown. Here, using a rat model of arterial insufficiency, we demonstrated that ultrasonic microbubble destruction generates angiogenesis, arteriogenesis, and enhanced skeletal muscle hyperemia. Furthermore, through the use of chimeric models in which wild-type mice were engrafted with BMDCs from CD18−/− and GFP+ mice, we found that US-MB–induced vascular remodeling involves the CD18-dependent recruitment, but not vascular incorporation, of BMDCs. Measurements taken shortly after US-MB treatment indicate that the treatment itself does not compromise perfusion in the short-term (supplemental Figure II), thereby excluding the possibility that arteriogenesis occurs in response to additional ischemia. Indeed, a modest vasodilatation occurs after ultrasonic MB destruction (supplemental Figure II), raising the possibility that elevated shear and circumferential wall stress could contribute to arteriogenic remodeling including collateral lumen diameter expansion and downstream capillary arterialization.

This study expands on our previous investigations of US-MB–induced arteriogenesis and enhanced hyperemia in the rat.17,19 First, we had previously tested the US-MB method in a model in which 1 of the 2 major feed arteries to the gracilis adductor muscle was occluded and shown that arteriogenesis occurs between regions of low and normal perfusion.19 Here, by using a model in which the femoral artery was occluded upstream of both major gracilis muscle feed arteries, we demonstrated that ultrasonic microbubble destruction may also stimulate arteriogenesis in a muscle entirely affected by arterial occlusion. Second, in that same previous study,19 US-MB treatment was applied concurrently with the placement of arterial ligations. The current study better represents the clinical presentation of vascular disease because we used a 2-week recovery period between arterial occlusion and treatment. It is unlikely that a slowly developing "gradual" femoral artery occlusion would have further enhanced clinical relevance because, after 2 weeks, endogenous changes in angiographic score are essentially the same for acute and gradual occlusions in the rat.23 Finally, by measuring hyperemia throughout the entire muscle, the statistical accuracy of the fluorosphere method was increased over our previous study.19 In the current study, the recommended minimum of 400 fluorospheres per tissue piece was easily achieved.

Our findings (Figure 1H) are supported by a recent study in which increased hindlimb blood flow was observed 4 weeks after ultrasonic microbubble destruction.21 More recently, ultrasonic microbubble destruction was used to deliver vascular endothelial growth factor (VEGF)16 plasmid to ischemic skeletal muscle.24 One experimental group from that study, in which a presumably inert GFP plasmid was delivered by ultrasonic microbubble destruction, is fairly comparable to our US-MB treatment group. Consistent with our results, a significant increase in blood volume, representing
enhanced arteriogenesis, was observed; however, no changes in resting perfusion occurred. Although this result appears to contradict our findings (Figure 1H), note that our measurements were made in the presence of a vasodilator. Thus, it is possible that differences in vascular tone account for this discrepancy. Indeed, in the absence of a vasodilator, we have also seen little to no change in blood flow following ultrasonic microbubble destruction.17

We have previously proposed that ultrasonic microbubble destruction elicits angiogenesis and arteriogenesis by recruiting BMDCs to treated tissue, where they may serve as paracrine growth factor sources or vascular cell precursors.17,19 In support of this hypothesis, other studies have indicated that components of inflammation may be stimulated by ultrasonic MB destruction.24,25 Furthermore, it has been shown that ultrasonic microbubble destruction elicits the recruitment of VEGF+ BMDCs to treated muscle22 and that increasing leukocrit before US-MB treatment enhances arteriogenesis; however, no “loss of function” studies have been performed to mechanistically prove that blocking BMDC recruitment abrogates US-MB–induced arteriogenesis. To this end, we modified the mouse hindlimb model used in our previous studies18 by adding femoral artery occlusions and using chimeric mice with BMDCs derived from CD18−/− mice. CD18 is the β2 integrin subunit which, along with the αL subunit (ie, CD11b), comprises Mac-1, the receptor for intracellular adhesion molecule-1 (ICAM-1). Because ICAM-1/CD18 interactions mediate firm adhesion during BMDC capture, the deletion of CD18 may be used to abrogate BMDC recruitment. Indeed, we found that, in CD18−/− chimeric mice, CD11b+ monocytes were not recruited to US-MB–treated muscles (Figure 4). Importantly, both arteriogenesis and angiogenesis were also blocked in CD18−/− chimeric mice. Although we anticipated only a moderate reduction of the arteriogenic response in the CD18−/− morph chimera, our measurements showed a surprising decrease in arteriogenesis to the level of the CD18+/− chimera (Figure 2), indicating that even the partial disruption of CD18 abrogates arteriogenesis. Consistent with our results, it has been shown that platelets activated by ultrasonic MB destruction release factors that upregulate endothelial P-selectin and ICAM-1 and an increase in the adhesion of BMDCs.21 Finally, we note that, even though the CD18+ cells that were
recruited to ultrasound microbubble–treated muscles were originally derived from bone marrow, it was possible that some CD18<sup>−/−</sup> cell fractions actually resided in nonnarrow compartments, such as the thymus or spleen, immediately before treatment.

Although the CD18<sup>−/−</sup> chimeric mouse studies demonstrate that BMDC recruitment via CD18 is required for this process, they do not elucidate the role of the recruited BMDCs in angiogenesis and arteriogenesis. Recent studies indicate that, depending on the extent of the stimulus, BMDCs may play important but functionally distinct roles in augmenting vascular remodeling. Although several studies have shown the direct incorporation of bone marrow–derived cells into remodeling vessels,<sup>26</sup>–<sup>29</sup> evidence to the contrary has emerged as well, demonstrating that these cells may not necessarily incorporate into vascular structures<sup>30,31</sup> and may serve mainly as paracrine sources of cytokine/growth factor production.<sup>32–34</sup> Through the use of GFP<sup>+</sup> chimeric mice, we observed the spatial location of BMDCs recruited to US-MB–treated tissues. After observing approximately 4000 microvessels from US-MB treated muscles in GFP<sup>+</sup> chimeric mice, we found no evidence for transdifferentiation or direct incorporation of BMDCs into the remodeling vasculature (supplemental Figure VII). When this result is considered in concert with our findings that US-MB treatment fails to stimulate arteriogenesis and angiogenesis in CD18<sup>−/−</sup> chimeric mice, we conclude that US-MB treatment elicits these responses via the recruitment of CD18<sup>+</sup> BMDCs to treated muscle but does not involve the transdifferentiation of BMDCs into vascular cells.

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Disclosures

Dr Klibanov is a shareholder of Targeson LLC.

References


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Supplemental Methods

Microbubble Preparation

As previously described,\textsuperscript{1,2} octafluoropropane gas (Flura, Newport, TN) was layered above a 1% solution of serum albumin in normal saline in a flask. To create MBs, the solution was sonicated for 30 sec with an US disintegrator (XL2020, Misonix, Farmingdale, NY) equipped with an extended 1/2" titanium probe.

Bone Marrow Transplantation Procedure

After exposure to lethal irradiation (12 Gy) (\(n=12\)), 6 to 8 week-old C57BL/6J mice were injected with \(2\times10^6\) bone marrow cells harvested from the tibias and femurs of donor mice. Donor mice included: 1) a WT C57BL/6J mouse, 2) a mouse whose cells, except hair cells and erythrocytes, ubiquitously express enhanced green fluorescent protein (EGFP) (C57BL/6-Tg[ACTB-EGFP]1Osb; Jackson Laboratory, Bar Harbor, ME), 3) a mouse with deficient but not absent expression of CD18 (CD18 hypomorphic mouse: B6.129S7-Itgb2\textsuperscript{tmm1Bay/J}; Jackson Laboratory, Bar Harbor, ME, Ref. 3), and 4) a mouse completely lacking CD18 expression (CD18\textsuperscript{−−} mouse: gift from Dr. Klaus Ley, Ref. 4).

Arterial Occlusion

Rats from Groups I-IV were anesthetized by an intraperitoneal (IP) injection of ketamine (80 mg\(\times\)kg\(^{-1}\)) and xylazine (8 mg\(\times\)kg\(^{-1}\)), while mice (Groups V-IX) were anesthetized by continuous isoflurane inhalation (2.5\% with 0.3-1.0 L/min of O\(_2\)). Using sterile techniques, bilateral femoral artery occlusions (AO) were administered to all animals with 6-0 silk sutures positioned on the femoral artery just distal to the femoral-epigastric-muscular branch trifurcation point. For a
schematic illustrating the occlusion location, please refer to Supplemental Data Figure I.

Laser Doppler Perfusion Imaging

Gracilis muscle perfusion was assessed in Group I animals by laser Doppler perfusion imaging (LDPI) (Lisca PIM) immediately after surgical exposure of the gracilis muscle, but before application of the US-MB treatment. Measurements were also made 2 and 24 hr after US-MB treatment. Animals were placed on a surgical heating pad and muscles were scanned at a resolution of 64×64 pixels to produce a color perfusion image. Mean voltage was quantified within the region of interest (i.e. the gracilis muscle), which was defined using a greyscale image of the scanned tissue. In turn, mean voltage was used to calculate the ratio of AO+US+MB muscle perfusion to AO+MB muscle perfusion. Animals were kept anesthesized between the US-MB treatment and the first LDPI measurement (2 hr), by a single 0.2 ml intravenous infusion of pentobarbital that was diluted 1:10 in 0.9% saline. The skin over each muscle was closed with absorbent sutures and animals were allowed to recover. Twenty four hours after US-MB treatment, animals were re-anesthetized by an IP injection of ketamine (80 mg×kg⁻¹) and xylazine (8 mg×kg⁻¹), gracilis muscles were exposed, and LDPI scans were repeated.

Fluosphere Blood Flow Measurements

Rats were re-anesthetized with an IP injection of ketamine and xylazine as described above, and gracilis muscles were surgically exposed and superfused with 37°C Ringer’s solution. For Group I, resting nutritive blood flow was measured immediately after the final LDPI scan, corresponding to 24 hours after US-MB treatment. For Groups II and III, hyperemia measurements were made 4 and 6 weeks after ligation, corresponding to 2 and 4 weeks after US-
MB treatment. Hyperemic conditions were created in Groups II and III by adding $10^{-4}$ M adenosine to the Ringer’s solution. Cannulas were placed in the carotid and mesenteric arteries. After 30 minutes of superfusion, 1.5 mL of 15 μm red fluospheres (Molecular Probes) was injected through the carotid artery over a 30 sec period. Fluosphere injection was followed by a 0.25 ml flush of isotonic saline. Starting at the time when fluosphere injection was initiated, blood was withdrawn by hand from the mesenteric artery for a duration of 90 sec. We have used this same volume of fluospheres in previous studies to measure absolute blood flow in gracilis muscle$^{2,5}$ and obtained values that match other published results,$^{6-8}$ therefore, we believe that the fluosphere injection itself had no significant influence on muscle perfusion.

Animals were then euthanized and gracilis muscles were fixed in their in situ positions by a 30 min superfusion of 4% paraformaldehyde in PBS. Fixed gracilis muscles were dissected free, weighed, and placed on microscope slides. The number of red fluospheres per muscle ($f_{M_{\text{red}}}$) was directly counted with a Nikon TE-300 fluorescence microscope and a ×20 objective. Meanwhile, the withdrawn blood volume was recorded, 7 ml of 2M ethanolic KOH containing a known quantity of 15 μm yellow-green fluospheres ($f_{R_{\text{yg}}}$) was added to the blood sample. Yellow-green fluospheres were added to control for any loss of fluospheres during processing. Forty-eight hours later, red and yellow-green fluospheres were extracted from the digested blood sample by centrifugation. Triplicate fluosphere counts were made for each sample by hemacytometer ($f_{B_{\text{red}}}$ = number of red fluospheres in processed blood sample; $f_{B_{\text{yg}}}$ = number of yellow-green fluospheres in processed blood sample) using epifluorescence microscopy. Gracilis muscle blood flow ($Q$) was then calculated as,
where $V$ is the volume of collected blood (ml), $t$ is blood the collection time (min), and $M$ is muscle mass (g).

**Immunohistochemistry Methods**

Whole gracilis muscles from rats and mice in Groups II-IX were incubated in 1:200 Cy3-conjugated monoclonal anti-smooth muscle (SM) $\alpha$-actin (clone 1A4; Sigma), washed in PBS-saponin, and imaged as whole mounts. Whole muscle montages were reconstructed from individual confocal fields-of-view and analyzed as described in the “Whole Muscle Image Analysis” section below. Group V-IX muscles that had already been immunolabeled for SM $\alpha$-actin as whole-mounts were then cryosectioned and incubated with 1:200 AlexaFluor488® (Molecular Probes) *Bandeiraea simplicifolia-I* (BS-1) lectin, 1:200 biotin-conjugated monoclonal anti-CD11b (clone M1/70; Serotec), or 1:100 biotin-conjugated monoclonal anti-CD18 (clone M18/2; BioLegend). After PBS-saponin washes, sections undergoing staining for CD11b and CD18 were incubated with 1:1000 AlexaFluor488®-conjugated streptavidin (Molecular Probes). Green fluorescent protein$^+$ (GFP$^+$) chimeric mouse muscle sections were also incubated with a fluorescently-tagged antibody against GFP (Molecular Probes) to enhance the GFP signal of bone marrow-derived cells (BMDCs). After PBS washes, sections were mounted for digital imaging.

**Whole Muscle Image Analysis**

From montaged reconstructions of whole-muscles from rats in Groups II and III, three sets of
measurements were made. First, we measured the ratio of smooth muscle (SM) \( \alpha \text{-actin}^+ \) microvessel area to the total \textit{en face} area of the gracilis muscle. Second, we measured the total number of >35 \( \mu m \) diameter SM \( \alpha \text{-actin}^+ \) microvessels intersecting 2 lines drawn perpendicular to the longitudinal muscle fiber direction and spaced equidistant from the proximal and distal muscle edges. Third, we measured the diameters of pre-selected order one and two arterioles (A1, A2) and venules (V1, V2). Order one vessels, which were defined based on their direct connection to the saphenous vessel pair, were measured halfway between the saphenous vessel pair and the first bifurcation within the muscle. Order two vessel measurements were then made on the second order vessels that were connected to the first order vessels via the first bifurcation within the muscle.

Images of SM \( \alpha \text{-actin} \) vascular networks within whole muscles from Group \( V-IX \) mice were analyzed by drawing 7 lines perpendicular to the muscle fiber direction and equally-spaced from the saphenous artery to the muscular branch. With the aid of Scion Image, the intersections of these lines with arteries and arterioles (>35 \( \mu m \) in diameter), as determined by SM \( \alpha \text{-actin} \) staining and morphology, were quantified for each specimen. In addition, arterioles were traced, and the vessel tracings were skeletonized, reducing these lines to one pixel width. The total number of line pixels was divided by the total number of pixels in the image, giving an effective arterial vessel length density measurement.

**Muscle Section Image Analysis**

Prepared sections were viewed with a Nikon TE-300 inverted microscope and a \( \times20 \) PlanFluor objective. Using a Bio-Rad Microradiance confocal scanner (Bio-Rad), digital images of every field of view within each section were obtained. Using Corel PHOTO-PAINT 11, fields-of-view
for each section were combined into a montage, creating a complete image of the muscle section. Scion Image was used to quantify vessel profiles positively marked with BS-1 lectin or SM α-actin, CD11b+ cells, CD18+ cells, GFP+ cells, muscle fibers in each section, muscle section area, and SM α-actin microvessel diameter. These measurements facilitated assessment of BS-1 lectin+ vessel profiles per muscle fiber, SM α-actin+ vessel profiles per muscle fiber, the diameter distribution of SM α-actin+ vessels, CD11b+ cells per muscle fiber, CD18+ cells per unit muscle area, and GFP+ cells per muscle fiber.
**Figure I.** Schematic of the experimental setup, including location of the femoral artery occlusion, as well as the application of ultrasound to the gracilis muscle.
Figure II. Ultrasonic MB destruction does not elicit a short-term reduction in resting muscle perfusion. A: Representative LDPI scans of rat gracilis muscles from AO+US+MB and AO+MB treated gracilis muscles. “S” denotes saphenous artery-vein pair. B: Ratio of LDPI perfusion in AO+US+MB-treated muscles to AO+MB controls. C: Absolute blood flows in AO+US+MB-treated and AO+MB control muscles 24 hours after ultrasonic MB destruction. No significant differences were found.
Figure III. Bar graph of total numbers of SM $\alpha$-actin$^+$ vessel profiles per muscle fiber grouped according to vessel diameter for WT and chimeric mice. Values are means ± SEM. *P<0.05 vs. AO+MB within same mouse type and AO+US+MB in CD18$^{-/-}$ chimeric mice. #P<0.05 vs. AO+MB within GFP$^+$ chimeric mice. **P<0.05 vs. AO+MB within CD18$^{-/-}$ chimeric mice. +P<0.05 vs. AO+US+MB within GFP$^+$ chimeric mice.
Figure IV. US-MB-induced angiogenesis depends on CD18 expression by BMDCs. A-D: Representative confocal images of BS-1 lectin+ vessel profiles in muscle sections from AO+US+MB and AO+MB hindlimbs of WT (A, B) and CD18−/− chimera (C, D) mice. Bar represents 50 µm. E: Bar graph of BS-1 lectin+ vessel profiles per muscle fiber for WT and chimeric mice. Values are means ± SEM. * P<0.05 vs. AO+MB in the same mouse type and vs. AO+US+MB group in CD18−/− chimera mice. # P<0.05 vs. AO+MB in GFP+ chimeric mice.
**Figure V.** Bar graph of CD18\(^+\) cells per unit area of muscle for AO+US+MB-treated WT chimeras and CD18\(^{−/−}\) chimeras. Data verify that the recruitment of CD18\(^+\) cells to treated muscle is greatly diminished in CD18\(^{−/−}\) chimeric mice. Values are means with standard errors. * P<0.05 vs. WT Chimera.
Figure VI. Representative confocal images of sections from AO+US+MB (A) and AO+MB (B) muscles of GFP⁺ chimera mice. Bar graph of GFP⁺ cells per muscle fiber (C). Values are means with standard errors. * P<0.05 vs. AO+MB.
Figure VII. Representative confocal images of GFP+ cells (green) and SM α-actin+ vessel profiles (red). Note that GFP+ cells are not incorporated into microvessels.
Supplemental References


