Microvascular remodeling and accelerated hyperemia blood flow restoration in arterially occluded skeletal muscle exposed to ultrasonic microbubble destruction

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The progression of atherosclerotic lesions in cardiovascular disease often leads to reduced resting and hyperemia blood flow to affected tissue. Current surgical interventions to restore flow cannot be applied to all patients. In those who are not candidates for surgical revascularization, therapeutic neovascularization may be an alternate option. Studies based on the introduction of cytokines (13, 27, 35), growth factors (25, 32, 36), growth factor genes (4, 26), or proangiogenic cells (11, 12, 15) to the site of reduced perfusion demonstrate the clinical potential of this strategy.

In addition to their primary role in generating diagnostic ultrasound images (16), contrast agent microbubbles have been used for therapeutic purposes, including targeted drug and gene delivery to the arterial vasculature (33, 34) and tissue (19, 21, 28, 30). We showed previously (29) that ultrasonic (1 MHz) microbubble destruction elicits arteriogenesis and enhanced hyperemia blood flow in normal rat skeletal muscle. In small animals, at frequencies below clinical levels, microbubble destruction creates pores in capillary walls (24, 29, 30). We hypothesize that this bioeffect recruits proangiogenic inflammatory cells, platelets, and/or marrow-derived stem cells to sites of microbubble destruction. Compared with other neovascularization strategies, this technique may allow visualization, targeting, and treatment with minimally invasive ultrasound.

We tested the hypothesis that ultrasonic microbubble destruction can restore hyperemia flow to skeletal muscle in a rat model of arterial insufficiency. One of the two gracilis muscle feed arteries was occluded while the other was left intact. Intravenously injected microbubbles were destroyed with 1-MHz ultrasound to stimulate arteriogenesis between the intact feeder and the low-perfusion zone. The deposition of arterially injected 15-μm fluo- rescence was used as a hyperemia flow index. Angiogenesis, the growth of new capillaries from existing vessels, and arteriogenes- isis, the formation and luminal expansion of arterioles and arteries, were measured with Bandeiraea simplicifolia (BS-1) lectin and smooth muscle (SM) α-actin staining, respectively.

METHODS

Microbubble preparation. Microbubbles were prepared by a standard perfluoropropane-exposed sonicated dextrose albumin approach (20). A 1% solution of serum albumin in normal saline was placed in a flask with a blanket of octafluoropropane gas (Flura, Newport, TN) above the aqueous phase. Microbubbles were generated by sonication (30 s) with an ultrasound disintegrator (XL2020; Misonix, Farming- dale, NY) equipped with an extended 0.5-in. titanium probe.

Ultrasound application. Animal studies were approved by the Animal Research Committee at the University of Virginia. All ani- mals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). The left jugular vein was cannulated for microbubble injection. The skin overlying the gracilis muscle to be treated was reflected back, ultrasound gel was placed over the muscle, and a 1-MHz unfocused transducer (Panametrics) with a diameter of 0.75 in. was held ~3 mm over the muscle. An ultrasound pulse was applied every 5 s for 2 min. Each pulse consisted of 100 consecutive 1-MHz sinusoids of 1-V peak-to-peak amplitude from a waveform generator (Tektronix AFG-310). The waveform signal was amplified by a 55-dB radio frequency power amplifier (ENI 3100LA). As measured with a hydrophone (model GL-0085; Specialty Engineering Associates), peak negative pressure was 0.5 MPa, corresponding to a mechanical index [peak negative pressure/ (frequency)1/2] of 0.5.

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Acute capillary disruption response. The effect of microbubble dose on the number of capillary disruptions per unit muscle mass was determined in Sprague-Dawley rats weighing between 270 and 280 g. Microbubbles (0.2 ml) in one of six concentrations ($n = 3$ rats/concentration) ranging from $2.5 \times 10^7$ to $3.0 \times 10^9$ microbubbles/ml, were injected through the jugular vein cannula, and ultrasound was applied to a gracilis muscle with the protocol described in Ultrasound application. The number of capillary disruptions per muscle was immediately counted with a Zeiss Makroskop with a fiber optic illuminator. The wet weight of the muscle was then measured to yield the total number of capillary disruptions per unit muscle mass.

Arterial occlusion and sham treatment protocols. Sprague-Dawley rats weighing between 250 and 280 g were randomly assigned to one of the four treatment groups designated in Table 1. Muscle specimens derived from these four treatment groups were defined according to the interventions the rat received [i.e., arterial occlusion (AO), sham surgery for AO (Sham), microbubble injection (MB), surgical intervention to position the transducer (Sur), ultrasound application (US), and no interventions (Norm)]. To eliminate any potential left-right data bias, these interventions were performed on alternating sides as the experiments proceeded. After anesthetization and placement of a jugular vein catheter, arterial occlusions were made with 6.0 silk at the sites indicated in Fig. 1. Arteries feeding Sham-treated muscles were freed from surrounding connective tissue but were not ligated. Group I animals were allowed to recover at this point. Group II and III animals were prepared for ultrasound treatment. For Group II animals, ultrasound was applied on one side to generate a AO+Sur+US-treated muscle. The transducer was then placed over the contralateral AO+Sur-treated muscle but not activated. For Group III animals, AO+MB+Sur+US-treated muscles were generated when microbubbles (0.2 ml at $2.0 \times 10^9$ microbubbles/ml) were injected intravenously and ultrasound was applied. This concentration of microbubbles was found to be the minimum dose that yielded the maximum number of capillary disruptions per unit muscle mass (Fig. 2). For AO+MB+Sur+US-treated muscles, the transducer was then placed over the contralateral muscle but not activated. The skin over each muscle was closed with absorbent sutures, and the animals were allowed to recover.

Hyperemia blood flow. Specimens were assigned for flow measurements and immunochemistry as described in Table 2. Flows were measured under hyperemia conditions to better isolate structural microvascular remodeling from short-term adjustments in vasoactivity. Animals were anesthetized as described in Ultrasound application, and gracilis muscles were exposed and superfused with 37°C Ringer solution containing $10^{-4}$ M adenosine for 30 min. A cannula was placed in the carotid artery and advanced to the aortic arch, and 1.5 ml of 15-μm red fluorospheres (Molecular Probes) were injected. We previously (31) used the same volume and concentration of fluorospheres to measure absolute flow in normal gracilis muscle. These values closely matched published results (5, 9, 10), verifying that the fluorosphere injection had no significant influence on flow. Group I muscles were processed for absolute flow measurements as previously described (31). Group II–IV animals were euthanized, and gracilis muscles were dissected in situ positions by a 30-min superfusion of 4% paraformaldehyde in PBS. Dissected gracilis muscles were placed on microscope slides, and the number of fluorospheres per field of view was counted in both the lateral and medial muscle regions (Fig. 1) with a Nikon TE-300 fluorescence microscope and a $\times 20$ objective.

Immunohistochemistry and specimen analysis. After quantification of fluorosphere deposition, lateral and medial muscle regions were cryosectioned. Cryosections were incubated overnight in 1:200 FITC-conjugated monoclonal anti-SM α-actin (clone 1A4; Sigma) and 20 μg/ml tetramethylrhodamine isothiocyanate-conjugated BS-1 lectin (Sigma) in PBS at 4°C.
Flow Restoration by Microbubble Destruction

RESULTS

Microbubble dose-capillary disruption response curve. Figure 2 depicts the effects of microbubble dose on the number of capillary disruptions per unit muscle mass. With doses of $4 \times 10^6$ microbubbles, a linearly proportional capillary disruption response was observed. However, at $\sim 40 \times 10^6$ microbubbles, an inflection point is reached and, although the curve still appears essentially linear, its slope decreases dramatically and the capillary disruption response is no longer proportional to microbubble dose. The curve plateaus at a dose of $400 \times 10^6$ microbubbles, indicating that this is approximately the minimum dose that will yield the maximum response. Therefore, this value was used throughout the remainder of the study.

Hyperemia flows. To provide a baseline characterization of the occlusion model without ultrasound or microbubble interventions, we measured absolute hyperemia flows in group I muscles (Fig. 3A). Acutely, the arterial occlusions reduced whole muscle hyperemia flow by 64%. At day 14, AO hyperemia flows remained 36% below Sham muscle levels, indicating that endogenous microvascular remodeling was not sufficient to return flow to baseline levels. In basic agreement with this result, compared with Norm muscle, statistically significant 30–40% reductions in hyperemia flow were observed in the lateral region of AO+MB+Sur muscles at days 7 and 14 (Fig. 3B). Medial region flow levels were also ~30–40% below Norm levels, but these differences were not statistically significant.

Muscles exposed to the complete ultrasound-microbubble treatment (AO+MB+Sur+US) exhibited significant increases in lateral region hyperemia flow compared with contralateral AO+MB+Sur control muscles at all time points (Fig. 3B).

Statistically, hyperemia flow was restored to Norm levels by day 7 in both regions of the AO+MB+Sur+US group, demonstrating that the treatment restored lateral region hyperemia flow well before it was restored by endogenous remodeling in the AO+MB+Sur control group at day 28. Moreover, in the AO+MB+Sur+US group, lateral region hyperemia flow continued to trend up until day 28, when the mean was within 5% of Norm levels. Hyperemia flows were measured in group II muscles to test whether microbubbles were required for flow enhancement and restoration. As shown in Fig. 3C, neither the lateral nor the medial muscle regions of AO+Sur+US muscles exhibited significant flow differences compared with AO+Sur contralateral controls. Moreover, no significant differences were observed when AO+Sur+US and AO+Sur specimens were compared with AO+MB+Sur.

Arteriogenesis. Independent of treatment, neither the gracilis muscle nor the distal hindlimb exhibited signs of necrosis. Figure 4, A–D, depicts SM α-actin labeling in representative cross sections 14 days after treatment. In the lateral region images, SM α-actin-positive vessel density in AO+MB+Sur+US muscles appears greater than in AO+MB+Sur controls. These results are quantified in Fig. 4E, which indicates that, compared with AO+MB+Sur, AO+MB+Sur+US muscles exhibit 40% and 28% increases in number of SM α-actin-positive vessels per fiber at days 7 and 14, respectively. The number of SM α-actin vessels per fiber in AO+MB+Sur+US muscles was also significantly greater than Norm at these time points. Furthermore, in the medial region of AO+MB+Sur+US muscles, the number of SM α-actin vessels per fiber was increased over that in AO+MB+Sur muscles at days 14 and 28. No differences were seen between AO+MB+Sur and Norm muscles, indicating that the occlusion alone did not elicit significant new SM α-actin-positive vessel formation. At day 14, the number of >30-μm-diameter arterioles in the medial region of AO+MB+Sur+US muscles was more than twofold greater than in AO+MB+Sur muscles (Fig. 4F). This value remained ~60% above that in AO+MB+Sur muscles at day 28. Compared with Norm muscles, the number of >30-μm-diameter arterioles per fiber was considerably elevated at all time points for both AO+MB+Sur and AO+MB+Sur+US muscles (Fig. 4F). We believe that these 5- to 10-fold differences between Norm muscles and the other groups are primarily caused by the baseline distribution of arteriolar diameters. As we showed previously (31), 10- to 30-μm arterioles are abundant in normal gracilis muscle, but >30-μm arterioles are scarce. Thus, when a large percentage of these smaller arterioles undergo luminal expansions of 10–20 μm, dramatic increases occur in the >30-μm category.

Table 2. Assignment of specimens for flow measurements and microvessel labeling

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<th>Group</th>
<th>Absolute Flow</th>
<th>Fluosphere Counting</th>
<th>Microvessel Labeling</th>
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Values are numbers of specimens assigned to each group.
Angiogenesis. Measurements of number of capillaries per fiber were made from BS-1 lectin-stained cross sections (Fig. 5). As illustrated in Fig. 5, A–D, and quantified in Fig. 5E, the lateral region of the AO+/MB+/Sur group exhibited a substantial, but transient, angiogenesis response. Specifically, at day 7, the number of capillaries per fiber was increased by 47% over the lateral region of AO+/MB+/Sur muscles and by 81% over Norm muscles. However, these values were not significantly elevated over AO+/MB+/Sur or Norm muscles at days 14 or 28. In the medial region of AO+/MB+/Sur+US muscles, the number of capillaries per fiber was increased over that in AO+/MB+/Sur muscles by 50% and 38% at days 7 and 14, respectively. By day 28, no differences in the number of capillaries per fiber were observed.

DISCUSSION

The major finding from this study is that microbubble destruction with 1-MHz ultrasound may be used to accelerate the restoration of hyperemia blood flow to skeletal muscle affected by arterial occlusions. Arteriogenesis occurred at sites both proximal and distal to an occluded feed artery, consistent with the hypothesis that accelerated flow restoration is due to an increase in the number and caliber of SM-coated collateral vessels connecting the patent feed artery to the region of low perfusion. Angiogenesis also occurred; however, this response diminished by day 28, when hyperemia flow was restored, thereby suggesting that capillary growth had a minimal effect on flow. Our results indicate that targeted microbubble destruction with ultrasound at subclinical frequencies may have potential as a means for stimulating therapeutic microvascular remodeling.

In many ways, the progression of the microvascular remodeling response observed in the current study parallels that observed by other investigators (6, 8). Here, we noted a sharp increase in capillary and SM-coated vessel density at an early time point (day 7), followed by the enhancement of hyperemia flow and a reduction in capillary density at a later time point (day 28). Although the occlusion sites were different, and no therapeutic intervention was used, a sharp increase in capillary density is first observed in rabbit hindlimb skeletal muscle after femoral artery occlusion (6). This angiogenesis response subsides, however, only 5 days after the intervention.

Fig. 3. A: absolute hyperemia flows in AO† and Sham-treated muscles. *Significantly different (P < 0.05) from Sham group at same time point. B and C: gracilis muscle hyperemia blood flows as assessed by fluosphere deposition. L, lateral region; M, medial region. Significant differences (P < 0.05): from lateral (*) and medial (**) regions of AO + MB + Sur group at same time point; from lateral region of Norm group (†).
Fig. 4. A–D: confocal images of smooth muscle (SM) α-actin-labeled microvessels in lateral (A, C) and medial (B, D) regions of AO+MB+SUR+ultrasound application (US) (A, B) and AO+MB+Sur (C, D) muscles. Images were taken 14 days after treatment. Bar, 100 μm. E: number of SM α-actin-positive vessels per fiber in Norm and lateral and medial regions of AO+MB+Sur+US and AO+MB+Sur muscles. Significant differences (P < 0.05): *from lateral region of AO+MB+Sur at same time point and Norm; **from medial region of AO+MB+Sur. F: number of >30-μm-diameter arterioles per fiber from AO+MB+Sur+US, AO+MB+Sur, and Norm muscles 7, 14, and 28 days after treatment. **Significantly different (P < 0.05) from medial region of AO+MB+Sur muscles.
with the return of capillary density to normal levels, the addition of angiographically visible collateral vessels becomes more pronounced. This arteriogenesis response, which continues for a considerable duration, is thought to be the primary mechanism for flow restoration (6, 8). Consistent with this hypothesis, our data show that hyperemia flow restoration occurred after capillary density had returned to normal, when the number of 30-μm-diameter arterioles per fiber was significantly above that in Sham control muscles in the medial region. It is important to note that the number of 30-μm-diameter arterioles per fiber in the lateral region was never significantly greater than that in Sham muscles. Moreover, in apparent contrast to previous studies (6, 8), this quantity trended down from day 7 to day 28 in both the Sham and treated groups. This apparent discrepancy may be due to the different stimuli used or to the fact that the vessels examined here (ranging from 30 to 100 μm in diameter) are angiographically invisible and, therefore, not as well studied with regard to arteriogenic remodeling. Indeed, it is probable that the arterioles bridging the capillaries and the collateral arteries exhibit unique remodeling behaviors. Nonetheless, the fact that lateral region hyperemia flows increased in concert with the number of larger-diameter arterioles in the medial region emphasizes the importance of arteriogenesis distal to the arterial occlusion. Finally, although it may be reasonable to speculate that high-flow collateral vessels supplying tissue regions near the occlusion site remain patent, their longevity remains to be studied.

Other topics for future studies will be the physiological, cellular, and molecular mechanisms that elicit remodeling.
both this study and a previous study (31), control experiments indicated that neither microbubble injection alone nor ultrasound application alone was capable of enhancing remodeling and flow. This suggests that, at this particular frequency and power, the interaction of microbubbles with ultrasound is required. However, aside from this observation, little is known of the remodeling mechanisms. Capillary disruption likely alters local hemodynamics, tissue oxygenation, and capillary permeability to growth factor diffusion, and all of these factors could stimulate angiogenesis and/or arteriogenesis. Furthermore, even though microbubbles must be present, disruption of the capillary wall may not be the sole stimulus. Inertial microbubble cavitation also creates thermal, shock wave, and sonoporation effects that could subsequently stimulate microvascular remodeling.

Another potential mechanism is the recruitment of inflammatory cells, platelets, and/or marrow-derived stem cells to sites of microbubble destruction. It is well known that inflammatory cells and cytokines, as well as platelets, may be used to stimulate therapeutic remodeling (11, 12, 15). Clinically, the cytokine granulocyte-macrophage colony-stimulating factor enhances collateral flow in the myocardium of patients with extensive coronary artery disease (27). Monocyte chemotractant protein-1 (MCP-1) administration is also an effective means for stimulating arteriogenesis (13, 35). However, when MCP-1 is given to apolipoprotein E-knockout mice, collateralization is accompanied by neointimal formation and plaque development (35). Obviously, this is an important side effect that must be considered in all therapeutic neovascularization strategies. Although we have yet to address this issue, we have observed directly (29) that vessel wall disruption is limited to only capillaries; therefore, we speculate that large vessels are not affected. Clearly, future studies will be needed to confirm this speculation.

A number of technical issues must also be resolved if this technique is to find clinical utility. Transducers capable of imaging at one frequency and destroying microbubbles at another will be required for locating and targeting intervention sites. Additionally, the bioeffect appears limited to small species (1, 18). This may be due to a number of reasons, including smaller capillary diameters and reduced ultrasound power attenuation. Clearly, for the method to be adapted to humans, a much better understanding of the physical interactions between low-frequency ultrasound, inertially cavitating microbubbles, and the capillary wall is needed. Finally, because the microvascular remodeling response will only occur when intravascular microbubbles are present, the method may not be effective within tissue regions lacking adequate perfusion. Instead, its use may be limited to the borders of perfusion deficit zones. However, even with this potential limitation, the current study demonstrates that microbubble destruction at the transitional area between low- and adequate-perfusion zones may be beneficial.

One advantage of our experimental model was that we could examine both flow and remodeling in tissue regions defined by their proximity to the arterial occlusions in a single specimen. However, because these tissue regions had small volumes, flow measurement errors caused by low total fluosphere entrapment were a concern. We addressed these potential errors in several ways. First, we replaced our standard protocol for fluosphere processing and fluorescence measurements (31) with direct fluosphere counts in the regions of interest, thereby eliminating errors associated with fluorescence retrieval. Similar methods, in which direct counts of fluospheres are made by microscopy, have been successfully used to measure blood flow in the pancreas (3, 17) and regions of the kidney (14). For simplicity, and because comparisons between AO+MB+Sur and AO+MB+Sur+US muscle regions did not require absolute measurements, we used fluosphere deposition as an index of hyperemia flow for this portion of the study. Second, we compared the day 14 fluosphere count difference between AO+MB+Sur and Norm muscles (30–40%) to the day 14 absolute flow difference between AO and Sham muscles (36%). Aside from microbubble injection, which we have shown to have no effect on flow in normal muscle (31), these interventions are identical; therefore, the close agreement of these flow differences supports the measurement. Third, it has been shown that using smaller numbers of spheres does not cause a systematic over- or underestimation of flow. Instead, more experiments are simply needed to demonstrate the significance of any given difference under these conditions (2). Indeed, using appropriate statistical testing, we were able to show the significant differences in Fig. 3. Finally, other studies have shown that, when biological differences dominate measurement error, accurate flow measurements can be obtained by using smaller numbers of spheres (7).

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