Vascular recruitment in skeletal muscle during exercise and hyperinsulinemia assessed by contrast ultrasound

DANA DAWSON,1 MICHELLE A. VINCENT,2 EUGENE J. BARRETT,2 SANJIV KAUL,1 ANDREW CLARK,2 HOWARD LEONG-POI,1 AND JONATHAN R. LINDNER1
1Cardiovascular Division and 2Division of Endocrinology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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Abstract
Recent advances in contrast ultrasound imaging suggest that this technique may be used to rapidly assess skeletal muscle microvascular responses in patients. Vascular recruitment in skeletal muscle during exercise and hyperinsulinemia was assessed noninvasively by contrast-enhanced ultrasound. Exercise and hyperinsulinemia increased capillary blood volume (capBV), whereas hyperinsulinemia alone did not significantly alter capBV, capillary blood flow, or microvascular resistance vessels. The number of perfused capillaries (Ncap) in skeletal muscle can change in response to various physiological stimuli. Increases in Ncap during skeletal muscle exercise are thought to enhance capillary surface area for oxygen exchange and to decrease oxygen diffusion distance. The extent of capillary recruitment in skeletal muscle remains uncertain because previous studies have used techniques that provide a measure of total blood volume, most of which resides in larger conduit and resistance vessels. Hence, capillary responses to various physiological stimuli have been difficult to assess in intact skeletal muscle due to a paucity of noninvasive methods capable of quantifying capillary blood volume (capBV).

Methods
Animal preparation. The study protocol was approved by the animal research committee at the University of Virginia. Twenty-two male Sprague-Dawley rats (230–280 g) were anesthetized with pentobarbital sodium (50 mg/kg), and body temperature was maintained with a heating lamp. Catheters were placed in a carotid artery for blood pressure monitoring and blood sampling and in both jugular veins for administration of contrast ultrasound. Exercise increases both capillary blood volume and blood flow, measured by a flow probe, increased with both contractile exercise and insulin. Contractile exercise increased capBV more than twofold and V\textsubscript{RBC} fivefold. Insulin also increased capBV more than twofold in a dose-dependent fashion but did not significantly alter V\textsubscript{RBC}. Saline infusion did not significantly alter capBV, V\textsubscript{RBC}, or femoral artery blood flow. We conclude that physiological changes in skeletal muscle capillary perfusion can be assessed in vivo with the use of contrast-enhanced ultrasound. Exercise increases both V\textsubscript{RBC} and capBV, whereas hyperinsulinemia selectively increases only capBV, which may enhance skeletal muscle glucose uptake.

Address for reprint requests and other correspondence: J. R. Lindner, Box 800158, Cardiovascular Division, Univ. of Virginia Medical Center, Charlottesville, VA 22908 (E-mail: jlindner@virginia.edu). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
tion of insulin, glucose, and microbubbles. An ultrasonic flow probe connected to a flowmeter (T106, Transonic) was placed on the exposed femoral artery of the study hindlimb. Contrast-enhanced ultrasound. The adductor magnus and semimembranosus muscles of the hindlimb were imaged in short axis with a linear-array transducer connected to an ultrasound system (L7-4 transducer, HDI-5000, ATL Ultrasound). Pulse inversion imaging was performed at a centerline transmission frequency of 3.3 MHz. The mechanical index ([peak acoustic pressure] × [frequency]^{-1/2}), a measure of acoustic power, was set at 0.9 to destroy and image microbubbles with the same pulse (22). The acoustic focus was placed at the midmuscle level, and gain settings were optimized and held constant throughout the experiment. Data were recorded on 1.25-cm videotape by use of a S-VHS recorder (Panasonic MD830, Matsushita Electric).

Octafluoropropane microbubbles with an albumin shell (Optison, Mallinckrodt Medical) were infused intravenously at 120 μl/min. Images were first acquired during “continuous imaging” at a frame rate of 20 Hz to acquire background images. Because microbubbles within the ultrasound sector were destroyed by ultrasound imaging (see Characterization of the acoustic beam), intermittent imaging was performed using pulsing intervals (PI) from 1 to 20 s to allow incremental microvascular replenishment with microbubbles between each pulse until the volume within the beam was completely refilled (22). Several frames were acquired at each PI.

Averaged frames obtained during continuous imaging were digitally subtracted from averaged frames at each PI to eliminate signal from large intramuscular vessels characterized by a higher blood flow velocity. Analysis was also performed by digitally subtracting frames acquired at a PI of 1 s to further subtract noncapillary microvessels. The video intensity at each PI was measured from the background-subtracted images, and PI vs. video intensity data were fitted to the function, \( y = A (1 - e^{-\beta t}) \), where \( y \) is video intensity at PI \( t \), \( A \) is plateau video intensity, an index of microvascular blood volume, which is represented primarily by capBV during myocardial imaging (9), and \( \beta \) is the rate constant that provides a measure of average \( V_{RBC} \) (22, 23). Microvascular blood flow was then calculated by the product of \( A \cdot \beta \) (22, 23). Interobserver variability for these measurements was 6% (mean difference 0.1 ± 0.6) for \( A \) values, and 12% (mean difference 0.02 ± 0.06 s^{-1}) for \( \beta \).

In vitro characterization of the acoustic beam profile. In vitro experiments were designed to 1) confirm complete microbubble destruction with the ultrasound settings used in the in vivo experiments and 2) calculate the beam elevation at the acoustic focus. The latter information was used to determine the blood velocity of vessels eliminated from analysis by performing background subtraction with frames obtained at a PI of either 0.05 or 1 s. A segment of canine jugular vein was attached at either end to polyethylene tubing and immersed in a water bath. One section of tubing was placed in a calibrated roller pump (EP-1, Bio-Rad), and a reservoir was connected to the end of the tubing. The ultrasound transducer was placed in the water bath and oriented to image the vein in short axis with identical imaging parameters as in the in vivo experiments.

Initial images were recorded during infusion of 0.9% saline. A suspension of Optison (5 × 10^4 ml^{-1}) was then placed in the reservoir and infused through the system at a rate of 5 ml/min. Intermittent imaging was performed by varying the PI from every 50 to every 3,000 ms with an internal timer. Video intensity within the lumen of the vein was measured off-line, excluding specular reflection from the vessel wall. Three frames at each PI were averaged, and data from three different studies were averaged. The internal diameter (d) of the vein was measured by video calipers (average of 2 orthogonal planes), and mean velocity (v) was calculated by: \( v = 4Q/(\pi d^2) \), where \( Q \) is the flow rate determined by flow pump calibration.

Endothelial xanthine oxidase activity. Capillary xanthine oxidase activity, measured by single-pass metabolism of 1-methylxanthine (1-MX) to 1-methylurate, was used to assess changes in capillary surface area (18, 25). Allopurinol (10 μmol/kg) was administered intravenously to partially inhibit xanthine oxidase 5 min before a continuous intravenous infusion of 1-MX (0.4 mg⋅kg^{-1}⋅min^{-1}) was begun (18). Whole blood (300 μl) was obtained from the carotid artery and femoral vein and immediately centrifuged. Plasma (100 μl) was separated and added to 20 μl of 1 M perchloric acid and 12 μl of 2.5 M K_2CO_3. Plasma concentrations of 1-MX and oxyipurinol (reflecting allopurinol concentration) were measured using reverse-phase high-performance liquid chromatography (model 600E/486, Waters Technologies), as previously described, by use of a linear gradient elution technique (18, 24). The rate of 1-MX metabolism was calculated by the product of femoral artery blood flow and arteriovenous difference of 1-MX concentration.

In vivo protocol. The experimental protocol is shown schematically in Fig. 1. One hour after surgical preparation, baseline hemodynamics and femoral blood flow were recorded, and contrast-enhanced ultrasound of the adductor muscles was performed. These measurements were repeated just before completion of a 2-h infusion of either normal saline (n = 6), low-dose insulin (3 mU⋅kg^{-1}⋅min^{-1}; n = 6), or high-dose insulin (40 mU⋅kg^{-1}⋅min^{-1}; n = 4) while systemic eu glycemia was maintained (18). Arterial and femoral venous blood samples were collected for measurement of glucose uptake. In the six saline-treated animals, electrodes were placed into the adductor tendons and connected to a pulse generator (5345, Medtronic). Muscles were stimulated at 2 Hz using 5- to 7-mA monophasic square-wave pulses 1 ms in duration. Hemodynamics and femoral blood flow were recorded, and contrast ultrasound was performed 2–3 min after initiation of electrostimulated contraction. In six separate animals, measurement of capillary endothelial surface area by 1-MX metabolism was performed after a 2-h infusion of saline and repeated 2–3 min after initiation of stimulated contractile exercise.

Statistical analysis. Data are expressed as means ± SD. Comparisons were made by using Student’s t-test (paired and unpaired) and repeated-measures ANOVA. Data were considered significant at \( P < 0.05 \) (two sided).

Insulin-treated

<table>
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Saline-control and exercise

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1-MX, 1-methylxanthine measurement.
RESULTS

Characterization of the acoustic beam. Contrast-enhanced ultrasound images and video intensity data from the vessel lumen in the in vitro experiment are illustrated in Fig. 2. The mean video intensity measured during infusion of microbubbles while imaging at PI of 50 ms (20 Hz) was similar to that measured before contrast infusion (14 ± 4 vs. 14 ± 3), confirming complete microbubble destruction with the ultrasound settings used in this study. During infusion of microbubbles, video intensity increased progressively with prolongation of the PI until an interval of ~1.5 s was reached. The relation between PI and video intensity was slightly curvilinear due to laminar rather than plug flow (Fig. 2). The beam elevation at the acoustic focus was estimated to be 2.4 mm by the product of flow velocity in the vein (1.6 mm/s) and the estimated time required to completely refill the ultrasound elevation (1.5 s). On the basis of these measurements, we assumed that background subtracting-averaged frames acquired during continuous imaging at a frame rate of 20 Hz in the in vivo protocols would completely exclude from analysis vessels with a mean velocity greater than ~4.8 × 10⁻² m/s (calculated by dividing the elevation by 0.05 s). These velocities are characteristic for arterioles that are greater than ~100 μm in diameter, although there is significant heterogeneity in the velocity-diameter relation (5). Similarly, use of images acquired at a PI of 1 s as background would exclude vessels with a mean velocity greater than ~2.4 × 10⁻³ m/s, which eliminates signal from almost all noncapillary microvessels, as well as a fraction of the capillary signal (4, 5, 7).

Effect of exercise and insulin on capillary perfusion. In the in vivo studies, mean heart rate and arterial blood pressure did not vary significantly in the different treatment groups. Compared with baseline measurements, saline infusion did not alter skeletal muscle microvascular blood volume (measured using 20 Hz imaging as background), V_{RBC}, or femoral artery blood flow (Fig. 3). In these same animals, stimulated contractile exercise produced an approximately twofold increase in microvascular blood volume and a fivefold increase in mean V_{RBC}, accounting for a marked increase in femoral artery blood flow (Fig. 3). Examples of contrast-enhanced ultrasound images from the hind-limb adductor muscles and corresponding PI vs. video intensity curves from a rat at baseline and during contractile exercise are illustrated in Fig. 4. Compared with baseline, contractile exercise resulted in a more rapid rate constant (β values of 0.10 vs. 0.45) and a higher plateau video intensity (A values of 6.1 vs. 14.1), indicating greater V_{RBC} and microvascular blood volume, respectively.

Infusion of insulin did not significantly alter V_{RBC} but increased microvascular blood volume, the extent of which was greater for high- compared with low-dose insulin. Femoral artery blood flow also increased in response to insulin but was similar for both doses of insulin (Fig. 5). The dose-related increase in microvascular blood volume in insulin-treated animals was associated with greater (P < 0.05) mean glucose uptake, which was also dependent on insulin dose (0.1 ± 0.1 μmol/min in saline-treated controls vs. 0.7 ± 0.2 and 2.0 ± 0.7 μmol/min for low- and high-dose insulin, respectively). Examples of contrast-enhanced ultrasound images and corresponding PI vs. video intensity curves from a rat at baseline and at the completion of an infusion of insulin at 3 mU·kg⁻¹·min⁻¹ are illustrated in Fig. 6. Low-dose insulin infusion increased the plateau video intensity (A values of 3.9 vs. 12.3) without changing the rate constant (β value of 0.10), indicating an increase in microvascular blood volume without a change in V_{RBC}.

To derive capBV, alternate analysis of contrast-enhanced ultrasound data was performed by digitally subtracting frames acquired at a PI of 1 s (1 Hz) rather than at 0.05 s (20 Hz) to further eliminate signal from
essentially all noncapillary microvessels. In insulin-treated animals, subtracting frames at the longer PI resulted in small, nonsignificant decreases in the mean \( A \) values at baseline (3.8 ± 1.2 vs. 3.3 ± 2.5), during low-dose insulin (10.2 ± 2.6 vs. 8.4 ± 2.1) and during high-dose insulin (14.1 ± 1.4 vs. 13.6 ± 3.1). Therefore, insulin-mediated increases in total microvascular blood volume and \( \text{capBV} \) were calculated to be approximately the same. For saline-treated animals, subtracting frames at a PI of 1 s rather than 0.05 s resulted in a significantly lower mean \( A \) value only during the exercise stage (13.2 ± 4.0 vs. 10.4 ± 1.9, \( P < 0.05 \)). Consequently, the \( \text{capBV} \) increased only 1.5-fold in response to exercise.

The degrees of change in mean \( V_{\text{RBC}} \) with insulin or with exercise were similar when PI values were of 50 ms and 1 s used as background, indicating that the percent increase in \( V_{\text{RBC}} \) was similar in capillaries and the total microcirculation.

Illustrated in Fig. 7 are the percent changes in femoral artery blood flow and microvascular blood flow (calculated by the product \( A \cdot \beta \)) during exercise or after low-dose insulin infusion. For either stimulus, changes in femoral artery blood flow did not increase to the same degree as skeletal muscle microvascular blood flow. This disparity can be partially explained by the fact that skeletal muscle (or the specific skeletal muscle groups being stimulated during exercise) accounts for only part of the total flow distribution for the femoral artery.

**Effect of exercise on 1-MX metabolism.** Changes in microvascular blood volume measured by contrast-enhanced ultrasound were compared with changes in microvascular endothelial surface area, measured by activity of xanthine oxidase present in capillary endothelial cells. These comparisons were made in animals undergoing contractile exercise. Arterial oxyxynol concentrations at baseline and after initiation of contractile exercise were not significantly different (8.6 ± 2.2 vs. 10.0 ± 1.6). Increases in capillary surface area, measured by 1-MX metabolism, in animals undergoing exercise were similar to increases in microvascular blood volume determined by contrast-enhanced ultrasound (Fig. 8).

**DISCUSSION**

Contrast-enhanced ultrasound imaging performed during a steady-state blood pool concentration of microbubbles provides information on the two primary components of microvascular perfusion, \( V_{\text{RBC}} \) and microvascular blood volume (22). Assessment of these parameters as a means to quantify myocardial and renal perfusion has been explored with contrast-enhanced ultrasound in animal models (2, 22, 23) and in humans (13, 15). In the present study, we have demonstrated for the first time that physiological changes in skeletal muscle \( V_{\text{RBC}} \) and \( \text{capBV} \) can be assessed in vivo with the use of contrast-enhanced ultrasound imaging.
During skeletal muscle work, large increases in oxygen consumption must at least partially be met by increased red cell flux through tissue because enhancement of oxygen extraction is limited (4, 6, 7, 10, 19). Increased red cell flux can be achieved by increasing capillary $V_{\text{RBC}}$. Electrically stimulated muscle contraction has been shown in animal models to increase $V_{\text{RBC}}$ in skeletal muscle by $\geq 300\%$ (4, 6, 20). However, the methods used to measure these changes are complex and too invasive to be applied in human studies. In the current study, we noninvasively assessed skeletal muscle $V_{\text{RBC}}$ during contractile exercise with the use of contrast-enhanced ultrasound and found changes comparable to those found previously (4, 20). Increases in flow can also occur by increasing $N_{\text{cap}}$, which may also serve to decrease oxygen diffusion distance. Muscle stimulation similar to that used in our study has been shown to increase $N_{\text{cap}}$ by 35–100% depending on whether intravital microscopy or freeze-clamp histology techniques were used (4, 6, 7, 20). In the present study, changes in capBV measured with contrast-enhanced ultrasound suggest that the extent of capillary recruitment in response to exercise may even exceed 100%.

Increases in skeletal muscle blood flow may also occur in response to hormonal stimuli such as hyperinsulinemia (1, 3, 12, 14, 17, 18). The vasoactive properties of insulin may facilitate glucose delivery to skeletal muscle, which is responsible for $>80\%$ of glucose disposal. On the basis of Renkin models of homogeneous capillary flow and limited endothelial glucose permeability, increases in $V_{\text{RBC}}$ would be expected to have a minimal effect on glucose disposal, since the glucose diffusion capacity of individual capillaries can be enhanced only to a small degree (3, 11). Alternatively, changes in capillary density would have a much greater impact on skeletal muscle glucose disposal, because uptake is governed more by capillary surface area than by capillary permeability (3). In accord with these concepts, we observed that physiological hyperinsulinemia (low-dose infusion of 3 mU kg$^{-1}$ min$^{-1}$ in the rat) (21) did not alter $V_{\text{RBC}}$ but did increase capBV to a similar extent as did exercise. Moreover, greater increases in capBV in response to high-dose supra-physiological compared with physiological insulin were
resides in microvessels, of which only approximately half of the blood volume in resting skeletal muscle was underestimated by these techniques, because only one-third of total vascular rather than microvascular blood volume. Accordingly, the extent of capillary recruitment may be underestimated in previous studies, which may have been solved only by detailed analysis of the relationship between the vascular and metabolic actions of insulin. The microvascular effect of insulin to recruit capillaries in skeletal muscle, which may have been underestimated in previous studies, may be more critical to substrate exchange and glucose disposal than changes in total blood flow. This issue will likely be solved only by detailed analysis of the relationship between capillary perfusion and substrate exchange in muscle in response to insulin.

In summary, this is the first study to quantify changes in skeletal muscle perfusion with contrast-enhanced ultrasound. This noninvasive imaging technique may potentially be applied to assess metabolic regulation of skeletal muscle perfusion and responses to physiological stimuli in humans. Moreover, it may provide valuable insight into the vascular actions of insulin, an indicator of capillary endothelial surface area, in rats at baseline and during contractile exercise. Bolded text indicates significant differences compared with measurements made at baseline.

The insulin-mediated increases in capBV measured in the current study exceed muscle blood volume changes estimated by indicator-dilution models (3) or by positron emission tomography (12, 17). Although these techniques have provided valuable insight into the vascular actions of insulin, they are somewhat limited by their inability to resolve microvascular blood volume alone.Indicator-dilution models estimate total extracellular volume, whereas positron emission tomography measures changes in total vascular rather than microvascular blood volume. Accordingly, the extent of capillary recruitment may be underestimated by these techniques, because only one-half of the blood volume in resting skeletal muscle resides in microvessels, of which only approximately one-fifth of vessels <200 μm is in capillaries (16).

In this study, we were able to eliminate signal from larger vessels by exploiting the blood velocity gradient from large to small vessels. Elimination of signal from larger vessels was not necessary in previous studies using myocardial contrast echocardiography because the vast majority of the myocardial microvascular volume at rest resides in the capillaries (9). Results from the in vitro protocol indicated that digitally subtracting frames obtained at a PI of 0.05 s eliminated signal from vessels with a velocity greater than ~48 mm/s, characteristic of vessels that are larger than ~100 μm (5, 25, 26). Analysis was also performed by background-subtracting images obtained at a PI of 1 s, which completely eliminated vessels with a velocity >2.4 mm/s, thus eliminating signal from most noncapillary vessels. In insulin-treated animals, background-subtracting frames at the longer PI resulted in only small changes in the blood volume, represented by the mean A value, which is expected since the majority of blood volume in <100-μm microvessels resides within the capillary compartment (16). Accordingly, changes in blood volume during insulin infusion, regardless of dose, were similar in extent when capBV and total microvascular blood volume was observed. In saline-treated animals, background-subtracting frames obtained at a PI of 1 rather than 0.05 s significantly decreases the mean A value only during exercise, resulting in a lower calculated percent change in capBV compared with total microvascular blood volume. This difference was most likely due to a marked increase in V̇_{RBC} during exercise, which would increase capillary signal at a PI of 1 s and, hence, would eliminate a greater proportion of the capillary signal. Therefore, the 1.5-fold increase in capBV with exercise calculated using a PI of 1 s as background probably slightly underestimated the true increase in N_{cap}.

To determine whether changes in the A value reflected changes in microvascular blood volume, we compared data obtained by contrast-enhanced ultrasound with measurements of capillary endothelial xanthine oxidase activity, an independent measure of microvascular surface area. Contrast ultrasound data on microvascular blood volume were used because we had reason to believe that capBV data might have slightly underestimated changes in N_{cap}. The extent of microvascular blood volume changes by the two techniques was similar, although these comparisons were limited by the technical inability to make both measurements simultaneously in the same animals.

Contrast-enhanced ultrasound can also provide a quantitative measure of microvascular blood flow by the product of capBV and V̇_{RBC} (or A·β) (22). In this study, we determined that exercise- or insulin-mediated increases in microvascular blood flow calculated in this manner were greater than changes in femoral artery blood flow. One likely explanation for this discrepancy is that the femoral artery supplies other hindlimb tissues not affected by adductor exercise or hyperinsulinemia. In insulin-treated animals, this finding is also congruent with the previously proposed notion that insulin-mediated increases in capillary perfusion can result from a redistribution of blood flow from “nonnutritive” to “nutritive” vascular pathways within muscle (18). The concept of flow redistribution within the muscle or different tissue components of the hindlimb is further supported by the lack of further increases in femoral artery blood flow for high- compared with low-dose insulin, despite further increases in capBV and microvascular blood flow.

We believe that the results of the current study offer important insight into the controversial topic of the relation between the vascular and metabolic actions of insulin. The microvascular effect of insulin to recruit capillaries in skeletal muscle, which may have been underestimated in previous studies, may be more critical to substrate exchange and glucose disposal than changes in total blood flow. This issue will likely be solved only by detailed analysis of the relationship between capillary perfusion and substrate exchange in muscle in response to insulin.
prove valuable for studying abnormal skeletal capillary responses in metabolic disorders, such as glucose intolerance and diabetes, and muscle-wasting diseases in humans.

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