A new method to study changes in microvascular blood volume in muscle and adipose tissue: real-time imaging in humans and rat

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Innovative Methodology

A new method to study changes in microvascular blood volume in muscle and adipose tissue: real-time imaging in humans and rat. Am J Physiol Heart Circ Physiol 301: H450–H458, 2011. First published May 27, 2011; doi:10.1152/ajpheart.01174.2010.—We employed and evaluated a new application of contrast-enhanced ultrasound for real-time imaging of changes in microvascular blood volume (MBV) in tissues in females, males, and rat. Continuous real-time imaging was performed using contrast-enhanced ultrasound to quantify infused gas-filled microbubbles in the microcirculation. It was necessary to infuse microbubbles for a minimum of 5–7 min to obtain steady-state bubble concentration, a prerequisite for making comparisons between different physiological states. Insulin clamped at a submaximal concentration (~75 μU/ml) increased MBV by 27 and 39% in females and males, respectively, and by 30% in female subcutaneous adipose tissue. There was no difference in the ability of insulin to increase muscle MBV in females and males, and microvascular perfusion rate was not increased significantly by insulin. However, perfusion rate of the microvascular space was higher in females compared with males. In rats, insulin clamped at a maximal concentration increased muscle MBV by 60%. Large increases in microvascular volume and perfusion rate were detected during electrical stimulation of muscle in rats and immediately after exercise in humans. We have demonstrated that real-time imaging of changes in MBV is possible in human and rat muscle and that the method is sensitive enough to pick up relatively small changes in MBV when performed with due consideration of steady-state microbubble concentration. Because of real-time imaging, the method has wide applications for determining MBV in different organs during various physiological or pathophysiological conditions.

Capillary recruitment; perfusion; blood flow; insulin; exercise

THE LINK BETWEEN MICROVASCULAR blood volume (MBV) and metabolism has been acknowledged since the classical studies by August Krogh demonstrating increased MBV in muscle during contractions (21). Still, measurement of MBV in vivo has been challenging, especially in humans. In rats, arteriovenous extraction of infused 1-methylxanthine (1-MX) by the endothelial enzyme xanthine oxidase has been used as a measure of MBV (26, 27). However, this technique has not been useful in humans because of insufficient metabolism of 1-MX (unpublished observations by the authors), and thus another method using contrast-enhanced ultrasound has also been applied to measure MBV in rats and humans (14, 35). This method is based on infusion of microbubbles and has so far relied on discontinuous echo recordings at different times after high-mechanical index (MI) ultrasound destruction of the microbubbles (14). With the use of this methodology, it has been demonstrated that insulin causes increased MBV in skeletal muscle and that this effect of insulin is impaired in states of insulin resistance (17, 22, 36). It is believed that part of the metabolic effects of insulin may be secondary to insulin’s effect to increase its own delivery as well as the delivery of glucose (32, 33) and that the inability of insulin to increase MBV in insulin-resistant states is partly responsible for metabolic insulin resistance (6, 11). It has been demonstrated that the effect of insulin to increase MBV does not rely on increased total muscle blood flow but rather on redistribution of flow from nonnutritive to nutritive microvessels in the muscle (13, 32, 33).

In the present study, we have now developed and evaluated continuous real-time imaging of MBV using contrast-enhanced ultrasound to detect and quantify gas-filled microbubbles in the microcirculation. Real-time imaging has a major advantage that it allows continuous determination of MBV and therefore allows determination of the dynamics of MBV. In addition, it allows easy determination of the time it takes to reach steady-state concentration of the infused microbubbles so that MBV can be determined while the concentration of microbubbles in the blood is constant. This is a prerequisite for valid and comparable measurements during different interventions. We have evaluated the method by determining the effect of insulin on MBV in leg skeletal muscle of both female and male human subjects and rat hindleg muscle. We have previously shown that females are more insulin sensitive than males (16), and, because of the greater subcutaneous tissue layers found in female subjects, we were able to determine MBV in both subcutaneous and muscle regions in the female subjects. Markedly increased muscle MBV was assessed by electrical stimulation in rats and by exercise in humans.

RESEARCH DESIGN AND METHODS

Human Experiments

Twenty healthy volunteers [body mass index (BMI) <25 kg/m²; 9 females and 11 males] matched in accordance to age and BMI (Table 1) were allocated to one of three different experiments: a one-step submaximal hyperinsulinemic-euglycemic clamp (experiment 1, n = 9 females and 7 males). In the male subjects, this was immediately followed by a second clamp step raising the insulin concentration to near-maximal effective insulin concentration (experiment 2). Experiment 1 was one-legged knee-extensor exercise (experiment 3, n = 5 males) on a separate day. All subjects gave written informed consent, and the study was approved by the Copenhagen Ethics Committee.

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### Table 1. Biometric parameters of the subjects

<table>
<thead>
<tr>
<th></th>
<th>Females (n = 9)</th>
<th>Males (n = 7)</th>
<th>Males (n = 5)</th>
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<tr>
<td>Age, yr</td>
<td>27 ± 1.5</td>
<td>26 ± 1.4</td>
<td>29 ± 2.8</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>64 ± 2.1*</td>
<td>75 ± 2.5</td>
<td>75 ± 3.3</td>
</tr>
<tr>
<td>Height, cm</td>
<td>169 ± 2.8*</td>
<td>177 ± 1.5</td>
<td>182 ± 2.0</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22 ± 0.5</td>
<td>24 ± 0.9</td>
<td>22 ± 0.6</td>
</tr>
<tr>
<td>LBM, kg</td>
<td>46 ± 1.1*</td>
<td>64 ± 1.1</td>
<td>64 ± 2.2</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>18 ± 1.2*</td>
<td>10 ± 1.7</td>
<td>10 ± 1.9</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>28 ± 0.2*</td>
<td>13 ± 1.9</td>
<td>14 ± 2.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects; BMI, body mass index; LBM, lean body mass. Different from men, *P < 0.05.

Animal Experiments

Male Wistar rats weighing 240–260 g were obtained from Taconic Farms (Greve, Denmark). Animals were housed at 20–22°C on a 12:12-h light-dark cycle and were provided with a standard laboratory chow diet and water ad libitum and then fasted overnight (experiment 4). The study was approved by the Danish Animal Experimental Inspectorate and complied with the European Convention for the protection of Vertebrate Animals used for Experiments and Other Scientific Purposes (Council of Europe 123, Strasbourg, France).

Euglycemic-hyperinsulinemic clamp. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt). A polyethylene cannula (PE-50; Intramedic) was inserted in the carotid artery for blood sampling and blood pressure monitoring. In both jugular veins, intravenous infusions of anaesthetics, insulin, glucose, and microbubbles were applied. A tracheotomy was performed to assist spontaneous respiration during the experiment. Anesthesia was maintained during the experiment by a constant infusion of aqueous pentobarbital sodium (0.5 mg·min⁻¹·kg⁻¹ body wt) via one of the jugular veins. Sixty minutes after completion of the surgery, basal blood samples were drawn, and the hyperinsulinemic-euglycemic clamp was initiated by a constant intravenous infusion of insulin (10 mU·min⁻¹·kg⁻¹) (Actrapid; Novo Nordisk) for 90 min. MBV was measured before and after 90 min insulin infusion.

Muscle contraction. After a 90-min clamp, two 27-gauge needles were inserted under the calcaneus communis tendon and through the skin above the tensor muscle of facia lata for electric stimulation (D345 Powerstim; Digitimer). Muscle contractions at 1 Hz and 15 volts were performed for 15 min during continued microbubble infusion. After 10 min of contractions, MBV was measured.

Control Experiments

To exclude that the observed effects of insulin were simply due to the elapsed time from the basal measurements to the insulin-stimulated measurements, control experiments were performed in which MBV was measured twice in the basal state 60 min apart in humans and 90 min apart in rats (similar to clamp time).

Measurement of the MBV

The MBV was measured with a contrast-enhanced ultrasound (CEU) technique both in human and rat skeletal muscle using a linear-array transducer connected to an ultrasound system (L9–3 transducer, iU22; Philips Ultrasound, Santa Ana, CA). In the human experiments, the transducer was fixed to the thigh using an in-house manufactured strap-on device keeping the transducer in the same place throughout the experiment, allowing for cross-sectional imaging of the vastus lateralis muscle (Fig. 1, A and B). In rats, the transducer was positioned over the left hindlimb and secured for the course of the experiment to image a cross section of the adductor magnus and semimembranosus muscles. Perfluorin lipid microspheres (Definity; Lantheus Medical Imaging) were activated by a vial mixer (Lantheus Medical Imaging) at 4,500 oscillations/min for 45 s. Microbubbles (1.5-ml suspension) were diluted to 20 ml in the human experiments and to 60 ml in the rat experiments with sterile saline and infused intravenously at a rate of 1.2–1.5 ml/min (humans) and 60 μl/min (rats) using a rotating syringe pump (Vue Ject, BR-inf 100; Bracco, Geneva, Switzerland) to ensure a homogenous microbubble solution. Based on the information by the manufacturer of maximally 1.2E+10 microbubbles per activated vial, the dilution to 20 ml, the infusion rate of 1.5 ml/min, the distribution space equal to plasma volume, and a measured half-life of 2.16 min in humans (see below), we calculate a maximum number of ~800,000 microbubbles/ml plasma at steady state using the formula.
The infusion rate of microbubbles, where $C_p$ is the steady-state microbubble concentration, $Q$ is the flow rate constant, and $V_d$ is the plasma volume. In comparison, the total number of red blood cells is $5 \times 10^{10}$/ml of blood in males. This means that, at steady state, there is $\sim 1$ bubble/6,000 red blood cells in blood.

In both humans and rats, real-time imaging was performed using a low mechanical index (MI) of 0.08, thereby allowing the microbubbles within the ultrasound beam to resonate without destruction. The acoustic intensity (AI) that is generated from the resonating microbubbles is proportional to the microbubble concentration in the area of interest (35). We confirmed this observation by adding increasing amounts of microbubbles to a water-filled beaker and recording the ultrasound signal, which increased linearly with increasing bubble concentration [Supplemental Fig. S1A (Supplemental data for this article may be found on the American Journal of Physiology: Heart and Circulatory Physiology website.)]. In addition, infusing microbubbles at increasing infusion rates resulted in a linearly increasing ultrasound signal in the muscle bed (Fig. S1B). A high MI of 1.20 was used at the beginning of each recording to destroy the microbubbles. Essentially all microbubbles are destroyed with the high-MI pulse, thereby allowing recording of the replenishment of the microbubbles in the vasculature within the ultrasound beam. As can be seen from Fig. S2, refilling of the vasculature led to an ultrasound signal at the same intensity as before destruction. Data were exported to quantification software (Q-lab; Philips) for analysis. Regions of interest (ROI) were drawn clear of connective tissue and large vessels for quantification.

$$C_p = \frac{Q}{K \times V_d}$$

has a thickness of 10 mm, a typical ROI would comprise a tissue volume of $1,357 \pm 227$, $3,081 \pm 510$, and $555 \pm 26$ mm$^3$ in female, male, and rat hindlimb muscle, respectively. ROIs in the subcutaneous adipose tissue were of a magnitude of $61 \pm 10$ mm$^2$, giving a tissue volume of $610 \pm 101$ mm$^3$ in females. The acoustic intensity (AI) obtained during the first 0.5 s in the basal and insulin-stimulated state and after the first 0.24 s during muscle contractions were averaged and subtracted from the AI recorded during the remaining seconds, thereby eliminating background noise and the contribution from rapid-filling vessels (i.e., arteries, veins, and large arterioles or venules). An ROI was drawn around a large vessel and quantified to determine that the large vessels were fully filled with microbubbles within 0.5 and 0.24 s and, furthermore, to ensure that the systemic arterial concentration of microbubbles was similar during the two microbubble infusions (basal and insulin stimulated or during muscle contractions/exercise). Supplemental Fig. S3 shows that the arterial bubble concentration was identical during the first and the second microbubble infusion. To exclude that changes in MBV, especially in the subcutaneous adipose tissue, were due to changes in skin temperature, skin temperature was measured using a surface electrode connected to a digital thermometer CTD85M (Ellab, Copenhagen, Denmark). Calculations were made in accordance to Wei et al. (35) where AI vs. time curves were fitted to the exponential function: $y = A[1 - e^{-B(t-t_0)}]$, where $t$ is time (s), $B$ the time used for background subtraction, $y$ is the acoustic intensity at any given $t$, $A$ is the plateau AI defined as MBV, and $B$ is the flow rate constant (1/s) that determines the rate of rise of AI (35). Because the ultrasound signal will vary from person to person depending on the distribution space of the bubbles, the half-life of the bubbles in the circulation, the amount of fascia, and subcutaneous adipose tissue and the vascular tone, the MBV is not an absolute value but an index of MBV. Within each individual, however, changes in MBV can be measured in real time.
All MBV data presented are based on background-subtracted images and graphed as the average of four 45-s imaging periods in the ROI following the microbubble destruction (Supplemental movie).

Blood Analysis

Human experiments: Blood glucose concentrations were measured on an ABL 800 FLEX (Radiometer Medical, Copenhagen, Denmark), and plasma insulin was measured using an enzyme-linked immunosorbent assay (Dako Cytomation).

Animal experiments: Blood glucose concentrations were measured on a HemoCue 201+ (HemoCue), and plasma insulin was measured using an enzyme-linked immunosorbent rat insulin assay (DRG Diagnostics).

Statistical Analysis

Data are presented as means ± SE and were analyzed on Sigma plot software (version 11; Systat Software, San Jose, CA). Within-subject comparison between measurements at baseline, during, and at the end of the experiment were performed using a one-way repeated-measurement ANOVA. The Holm-Sidak test adjustments for multiple comparisons were used to determine in which of the individual conditions the differences were significant.

RESULTS

Microvascular Blood Volume and Microvascular Perfusion Velocity

Establishment of steady-state microbubble signal. Infusion of Definity microbubbles at a constant rate of 1.2–1.5 ml/min in humans resulted in a gradual increase in signal after a lag phase of ~60 s (Fig. 2A). As shown in Fig. 2A, a plateau signal is achieved after an average of 5–7 min infusion at rest. Therefore, an infusion time of 10 min was routinely used before MBV recordings were performed to ensure that these were performed during steady state. This time to reach steady state is also in agreement with the knowledge that, during infusion at a constant rate, steady-state concentration is achieved after approximately five half-lives, and, as can be seen below, the half-life of the microbubbles was found to be ~2 min on average. During exercise, it was impossible to reliably record CEU because of large movements of muscle during knee extensions, and, consequently, it is not possible to know exactly when a new plateau is reached. However, it is likely to have been reached rapidly since total muscle blood flow increases within seconds of commencement of exercise (2). During electrical stimulation of rat muscle where movement is limited and CEU recording is possible during stimulation, a plateau was reached already after 1 min (Fig. 2C). When no electrical stimulation was employed, the initial plateau after commencement of microbubble infusion was reached after an average of 3–5 min (Fig. 2B) but again to ensure steady-state MBV recordings were performed after 10 min of microbubble infusion. Upon cessation of microbubble infusion, the signal decayed with an apparent half-time of 2.16 ± 0.24 min in humans (n = 5) and 1.76 ± 0.17 min in rats (n = 3) (Fig. 2, A and B).

Experiment 1. Insulin infusion of 1.42 mU·min⁻¹·kg⁻¹ for 60 min resulted in an increased MBV by 27% (P < 0.05) (Fig. 3, A and B) and 39% (P < 0.001) (Fig. 3, E and F) compared with baseline in the vastus lateralis muscle in females and males, respectively. Supplemental video movies 1 and 2 show the refilling of the microvascular volume after destruction of the microbubbles in the basal state and in the insulin-stimulated state. Supplemental Fig. S3 shows that the arterial bubble concentration was identical during the first and the second microbubble infusion, ensuring that the increase in MBV during insulin infusion was not because of differences in arterial concentration of microbubbles. The flow rate constant (B-value) did not increase significantly from 0.150 ± 0.01 and 0.079 ± 0.01 in the basal state to 0.173 ± 0.02 and 0.085 ± 0.01 during the insulin infusion in females and males, respectively. However, females had a significantly higher flow rate constant than males both at basal (P < 0.001) and during insulin stimulation (P < 0.01). Furthermore, at this insulin concentration, MBV was increased by 30% (P < 0.05) com-

Fig. 2. Representative presentation of the development of the contrast-enhanced ultrasound signal from muscle with time during and after discontinuation of microbubble infusion in a resting man (A) and a rat (B) and during muscle contractions in a rat (C). Note that the scale on the y-axis is different between humans and rats because of contractions in C. AI, acoustic intensity.
pared with baseline in the subcutaneous adipose tissue in females (Fig. 3, C and D). Interestingly, the shape of the refilling curve in adipose tissue was markedly less steep than in skeletal muscle, reflecting a slower flow rate as indicated by $/H9252$-values of 0.032 and 0.111 in adipose tissue and muscle tissue in the females, respectively (Fig. 3, C and D). To exclude that the increase in subcutaneous adipose tissue MBV was the result of an increase in skin temperature rather than an effect of insulin, skin temperature was measured under the ultrasound transducer before and after insulin infusion. No change in skin temperature was found (Table 2). Glucose infusion rate during the clamp at similar plasma insulin levels tended to be higher ($P\sim0.10$) in females than males when expressed per kilogram lean body mass (Table 2), as found previously (16).

Experiment 2. When insulin infusion was increased to 5 mU-min$^{-1}$-kg$^{-1}$ for 60 min, no further significant increase was seen in MBV or in the flow rate constant ($/H9252$) in the vastus lateralis (Fig. 4A and 4B) despite an increase in glucose infusion rate (Table 2).

Experiment 3. One-legged knee extensor exercise at 5 watts for 10 min induced an increase in MBV in the vastus lateralis muscle of 170% ($P\sim0.001$) compared with baseline (Fig. 4, C and D). Increasing the workload to 25 watts for 10 min further increased MBV by 50% ($P\sim0.001$) compared with the 5-watt workload and by 310% ($P\sim0.001$) compared with baseline (Fig. 4, C and D). As expected, the refilling curves during exercise were markedly steeper than at rest and during insulin infusion (Fig. 4C). Consequently, the flow rate constant ($/H9252$) was increased significantly to 0.694 $\pm$ 0.07 at 5 watts and further to 0.959 $\pm$ 0.20 at 25 watts.

Animal studies. In experiment 4, insulin infusion of 10 mU-min$^{-1}$-kg$^{-1}$ for 90 min increased MBV in the hindlimb muscles (Fig. 4, E and F). The microvascular flow rate constant ($/H9252$) was

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**Fig. 3.** Left: microvascular refilling curves after destruction of the microbubbles in female and male vastus lateralis muscle (A and E) and subcutaneous adipose tissue in females (C) at basal and after insulin infusion. Right: microvascular blood volume presented as the plateau value $/H251$ in the vastus lateralis muscle ($/H251$ and $/H251$) and subcutaneous adipose tissue ($/H251$) at basal and after insulin infusion. Different from basal, *$P < 0.05$ and †$P < 0.001$. A, plateau value; $/H9252$, flow rate constant. Curves and bar graph values are means of $n = 9$ (A–D) and $n = 7$ (E and F) experiments, and error bars are SE.
similar in both the basal and insulin-stimulated state (Fig. 4E). When electrical-stimulated muscle contractions were added to the insulin-stimulated muscle during continuous clamping, MBV increased by 70% (\(P < 0.001\)), which was 175% (\(P < 0.001\)) higher than baseline (Fig. 4, E and F). In addition, electrical stimulation significantly increased the slope of the refilling curve in parallel to the effect of exercise in humans (Fig. 4, C and E).

Control experiments performed without insulin infusion demonstrated that there was no independent time effect, since

![Table 2. Plasma glucose and insulin concentrations and glucose infusion rates during a euglycemic-hyperinsulinemic clamp in humans and rats](image)

**Table 2. Plasma glucose and insulin concentrations and glucose infusion rates during a euglycemic-hyperinsulinemic clamp in humans and rats**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time, min: 0</th>
<th>Time, min: 60</th>
<th>Time, min: 120</th>
<th>Time, min: 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>58 ± 3.1</td>
<td>62 ± 2.8</td>
<td>57 ± 3.8</td>
<td>62 ± 5.5*</td>
</tr>
<tr>
<td>Plasma insulin, pmol/l</td>
<td>23 ± 2.6</td>
<td>448 ± 19†</td>
<td>26 ± 4.6</td>
<td>469 ± 22†</td>
</tr>
<tr>
<td>Plasma glucose, mmol/l</td>
<td>5.0 ± 0.9</td>
<td>4.9 ± 0.9</td>
<td>5.1 ± 0.1</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>GIR, (\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1})</td>
<td>27.2 ± 3.5</td>
<td>27.0 ± 1.2</td>
<td>50.5 ± 2.8‡</td>
<td>24.7 ± 0.2</td>
</tr>
<tr>
<td>GIR, (\mu\text{mol} \cdot \text{kg} \cdot \text{LBM}^{-1} \cdot \text{min}^{-1})</td>
<td>37.7 ± 3.1</td>
<td>31.4 ± 1.3</td>
<td>58.8 ± 3.2‡</td>
<td>32.5 ± 0.4</td>
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<tr>
<td>Skin temperature, °C</td>
<td>31.6 ± 0.6</td>
<td>31.7 ± 0.6</td>
<td>32.2 ± 0.2</td>
<td>32.4 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. HR, heart rate; GIR, glucose infusion rate. Different from basal, *\(P < 0.05\) and †\(P < 0.001\). Different from 60 min, ‡\(P < 0.001\).
the refilling curves and the AI plateau levels were identical during the two microbubble infusions in the basal state in both humans and rats (Fig. 5, A and B).

Plasma insulin. In experiment 1, insulin infusion of 1.42 mU·min⁻¹·kg⁻¹ for 60 min increased plasma insulin concentration to 448 ± 19 pmol/l in females and to 469 ± 22 pmol/l in males (P < 0.001) (Table 2). In experiment 2, when insulin infusion was increased to 5 mU·min⁻¹·kg⁻¹ for 60 min, plasma insulin concentration increased to 1,910 ± 80 pmol/l (P < 0.001) (Table 2). In experiment 3, insulin infusion of 10 mU·min⁻¹·kg⁻¹ for 90 min increased plasma insulin concentration to 1,821 ± 77 pmol/l (Table 2).

Heart rate. In experiments 1 and 2, insulin infusion of 1.42 mU·min⁻¹·kg⁻¹ for 60 min did not affect the heart rate in females or males but increased to 62 ± 5.5 beats/min in males (P < 0.05) at the high insulin infusion rate compared with baseline (Table 2). In experiment 3, one-legged knee-extensor exercise increased the heart rate to 79.6 ± 3.6 and 90.4 ± 2.2 beats/min (P < 0.004) compared with basal during the 5- and 25-watt workload, respectively.

Femoral artery blood flow. In experiment 1, basal femoral artery blood flow was similar in females and males and averaged 440 ± 22 ml/min. Insulin infusion at 1.42 mU·min⁻¹·kg⁻¹ for 60 min did not affect femoral artery blood flow significantly and averaged 480 ± 35 ml/min during the insulin infusion in both females and males. There were no significant differences in basal and insulin-stimulated femoral artery blood flow between females and males. In experiment 2, when insulin infusion was increased to 5 mU·min⁻¹·kg⁻¹ for 60 min, femoral artery blood flow increased significantly to 590 ± 80 ml/min (P < 0.05).

In experiment 3, basal femoral artery flow was 394 ± 38 ml/min. One-legged knee extensor exercise at 5 watts for 10 min significantly increased arterial blood flow to 1,398 ± 141 ml/min (P < 0.001) compared with baseline. Increasing the workload to 25 watts for 10 min further significantly increased blood flow to 2,700 ± 252 ml/min (P < 0.001) compared with baseline and the 5-watt workload.

DISCUSSION

This is the first demonstration and evaluation of a method involving constant infusion of Definity microbubbles to measure changes in real-time MBV and the flow rate constant (β) in skeletal muscle and adipose tissue in humans and rats. We were able to demonstrate that MBV is increased in muscle during physiological increases in plasma insulin concentrations similarly in males and females and furthermore that MBV is also increased by insulin in subcutaneous adipose tissue. Although there was a tendency toward an increase in the flow rate constant (β), this did not reach statistical significance. However, females displayed an increased flow rate constant (β) compared with males both in the basal and in the insulin-stimulated state. Finally, large increases in muscle MBV and β were demonstrated during exercise in humans and electrical stimulation in rat muscle. Because all organs that can be reached by ultrasound in theory can be examined with this technique, it has wide applications for determining real-time changes in MBV during various physiological or pathophysiological conditions.

In the present study, we measured real-time MBV by taking advantage of continuous resonance of low-energy ultrasound of infused perfluor-en-filled phospholipid microbubbles (CEU) of a size (2–4 μm) (5) that easily enter open capillaries. CEU has been used before to demonstrate the vasodilatory properties of insulin in human and rat skeletal muscle (15, 19, 31), however, in the earlier studies, filling curves were constructed by discontinuous echo recordings at different filling times after high-MI ultrasound destruction of the microbubbles (13, 14, 31, 32). In contrast, we report here the use of low-MI ultrasound to monitor MBV in real-time imaging. We believe this method offers significant advantages compared with the previous methods, as discussed below.

It has previously been shown that MBV in muscle is increased by insulin in humans and rats by CEU (17, 18, 31), and, in rats, this has been confirmed by the 1-MX method in which 1-MX is infused intravenously and the clearance of 1-MX to 1-methyluracil by the endothelial enzyme xanthine oxidase provides a measure of capillary perfusion (10, 26, 31). Unfortunately, in humans, the activity of endothelial xanthine oxidase is apparently markedly lower than in rats because we have been unable to detect measurable arteriovenous extraction of infused 1-MX across the leg in humans (unpublished observations). An advantage of real-time imaging is that it allows definitive establishment of a constant imaging signal before filling curves are constructed and more accurate curve fitting because of the continuous collection of data for 45 s after microbubble destruction rather than a few selected time points that are collected over several minutes. In addition, in contrast to previous imaging techniques, real-time imaging allows recording of MBV in states of rapid transition of perfusion such as immediately after cessation of exercise in humans. Previous
reports using real-time imaging with another contrast agent (Sonovue; Bracco Diagnostics) may not have established steady state before measurements because of a short (5 min) infusion protocol (19, 20) or a bolus injection (13, 24, 30a) and therefore suffer from lack of steady state, making data interpretation complicated by assumptions about systemic equilibrium and washout kinetics, which probably vary from person to person.

It might be speculated that the increase in AI obtained with insulin or exercise is due to a faster flow rate during capillaries already perfused in the basal state rather than perfusion of an increased number of capillaries. A major argument against this assumption is the model experiment in which an increase in flow rate in preexisting tubes did not lead to increased AI, whereas, when more tubes were perfused, the AI increased (29). These experiments clearly indicate that the increase in AI with insulin and exercise must be due to an increase in the number of microvessels perfused.

Although some studies (3), including some of our own (30), have previously demonstrated increased total limb flow with insulin infusion resulting in physiological insulin concentrations, the increase in MBV by insulin in the present study at the first clamp step was obtained without any measurable increase in total femoral arterial blood flow. This is in accordance with most studies at this insulin concentration (7, 13, 15, 16, 28) and probably indicates that, at a physiological insulin concentration, the effect of insulin on total blood flow is trivial (37) and therefore probably at the detection limit. However, during the second step, which resulted in unphysiological near-maximal insulin concentrations, a small increase in blood flow occurred (Table 2). Thus, in the absence of an increase in total leg blood flow, it is likely that insulin causes a redistribution of flow from so-called nonnutritive flow channels to nutritive capillaries (12, 13). Nonnutritive flow channels are made up of shorter and possibly larger capillaries supplying the connective tissue, whereas the nutritive flow channels are made up of longer tortuous capillaries in intimate contact with the myocytes (9). This redistribution of muscle blood flow is thought to be of major benefit for the effect of insulin on glucose uptake (4, 12), and it has also been shown to be absent in obesity (11) and related states of insulin resistance like type 2 diabetes (36) and in Zucker rats (34). Of note is also that the increase in MBV with insulin was similar in females and males, which has not been previously specifically studied. Interestingly, females displayed a higher flow rate constant in muscles than males. Therefore, this effect might lead to a generally higher capillary flow rate than in men.

Another novel observation of this study was that insulin induced an increase in MBV in subcutaneous adipose tissue (Fig. 3, C and D). Due to the fact that the males in this study were lean, subcutaneous adipose tissue on the thigh was not present in sufficient amounts to allow CEU imaging. However, in the females, subcutaneous adipose tissue was present in sufficient amounts (8.6 ± 0.3 mm compared with 3.0 ± 0.3 mm in males when measured on the ultrasound image) for imaging, and this allowed us to demonstrate that insulin, in addition to its vasodilatory effects in muscle, also increases MBV in subcutaneous adipose tissue. Interestingly, the filling curves were much less steep in adipose tissue compared with muscle, indicating a lower flow rate constant (B) at this neutral temperature environment (24°C).

Exercise and electrical stimulation of rat muscle led to marked increases in MBV and perfusion velocity as well as a marked increase in femoral arterial blood flow, as expected. These data were collected mainly as a positive control demonstrating that the technique is able to detect expected large increases in MBV. Because of the movement of the leg during exercise, it is not possible to perform the CEU during exercise in humans, but measures in the present study were performed during the first 20 s after exercise termination. It was noticeable that the CEU signal was constant for the first 15–20 s after termination of exercise whereupon it decreased rapidly. This indicates that the MBV imaged immediately after exercise probably is a reliable value of MBV during actual exercise and demonstrates the value of imaging in real time. The existence of increased MBV during exercise was recently debated (8, 25). The present data add to the evidence that changes in MBV do take place during dynamic exercise in humans (36) and during electrical stimulation in rat muscle (14).

**Limitations and Strengths of the Technique**

The ultrasound signal will vary from person to person depending on the bubble infusion rate, distribution space of the bubbles, half-life of the bubbles in the circulation, amount of fascia and subcutaneous adipose tissue, and the vascular tone. Thus the MBV is not an absolute value but an index of MBV. However, within each individual, changes in MBV can be measured noninvasively in real time. In humans, the infusion time available is ~12–16 min/MB vial when infusing microbubbles at 1.5–1.2 ml/min. Because the first 10 min of infusion time are needed to obtain steady state, this means that actual measuring time per infusion is limited to 2–6 min depending on infusion rate. However, repeated infusions are possible, as we have performed in the present study. Furthermore, as discussed in the paragraph above, reliable measurements cannot be performed during dynamic exercise because of movement of the tissues being measured, but, since measurements are performed in real time, it is possible to record at the instant the movements stop thus probably obtaining data similar to values during exercise.

In conclusion, we have demonstrated that estimates of MBV can be made in real-time imaging in human and rat muscle, and we have confirmed that insulin increases MBV in muscle and demonstrate that this effect is similar in females and males. In addition, we show that insulin also increases MBV in subcutaneous adipose tissue. Finally, we demonstrate that the method also allows determination of MBV in muscle in response to exercise in humans and during electrical stimulation of rat muscle. Based on the use of contrast agents in stress echocardiography in 26,774 patients, it was argued that the technique is safe (1, 23). In agreement with this, it might also be mentioned that no complications were noted during or after microbubble infusions in the present study. The method has wide applications for determining changes in MBV in different organs during various physiological or pathophysiological conditions.

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