

Skeletal Muscle Microvascular Recruitment by Physiological Hyperinsulinemia Precedes Increases in Total Blood Flow

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Supraphysiological doses of insulin enhance total limb blood flow and recruit capillaries in skeletal muscle. Whether these processes change in response to physiological hyperinsulinemia is uncertain. To examine this, we infused either saline ($n = 6$) or insulin (euglycemic clamp, $3.0 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $n = 9$) into anesthetized rats for 120 min. Femoral artery flow was monitored continuously using a Doppler flow probe, and muscle microvascular recruitment was assessed by metabolism of infused 1-methylxanthine (1-MX) and by contrast-enhanced ultrasound (CEU). Insulin infusion raised plasma insulin concentrations by ~ 10 -fold. Compared with saline, physiological hyperinsulinemia increased femoral artery flow (1.02 ± 0.10 vs. $0.68 \pm 0.09 \text{ ml/min}$; $P < 0.05$), microvascular recruitment (measured by 1-MX metabolism [6.6 ± 0.5 vs. $4.5 \pm 0.48 \text{ nmol/min}$; $P < 0.05$] as well as by CEU [167.0 ± 39.8 vs. $28.2 \pm 13.8\%$; $P < 0.01$]), and microvascular flow velocity (β , 0.14 ± 0.02 vs. $0.09 \pm 0.02 \text{ s}^{-1}$). Subsequently, we studied the time dependency of insulin's vascular action in a second group ($n = 5$) of animals. Using CEU, microvascular volume was measured at 0, 30, and 90 min of insulin infusion. Insulin augmented microvascular perfusion within 30 min ($52.8 \pm 14.8\%$), and this persisted at 90 min ($64.6 \pm 9.9\%$). Microvascular recruitment occurred without changes to femoral artery flow or β . We conclude that insulin increases tissue perfusion by recruiting microvascular beds, and at physiological concentrations this precedes increases in total muscle blood flow by 60–90 min. *Diabetes* 51:42–48, 2002

At supraphysiological doses, insulin augments total skeletal muscle blood flow in humans (1,2). Some but not all (2,3) investigators have reported a similar action with insulin at physiological concentrations in humans (3–5) and animal models (6). In addition to dose, the duration of insulin exposure may be important to the enhancement of muscle blood flow, with the effect being greatest after several hours of steady-state hyperinsulinemia (2). The potential

importance of these vascular actions of insulin is underscored by the observation that *N*^G-monomethyl-L-arginine methyl ester can block insulin's action on total blood flow and concomitantly inhibit up to 30% of insulin-mediated glucose disposal (7).

While exploring insulin's vascular actions, we observed that high-dose insulin infusion ($10 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) increased rat hindlimb blood flow in vivo and also enhanced the conversion of exogenously infused 1-methylxanthine (1-MX) to 1-methylurate (1-MU) by endothelial xanthine oxidase (8). Because this enzyme is located on the capillary endothelial surface (9) these data indicated that insulin enhances the exposure of 1-MX to xanthine oxidase (8). To our knowledge, this was the first report providing experimental evidence for an action of insulin on muscle microvascular recruitment. We also observed that epinephrine infusions, which enhanced total hindlimb blood flow to a similar degree as insulin infusions, did not affect microvascular recruitment as measured by 1-MX metabolism (8). This suggested that total flow and microvascular recruitment could be dissociated. Subsequently, we reported that high-dose ($10 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) insulin enhanced the laser Doppler signal in rat muscle obtained using either surface scanning or an implantable laser Doppler probe (10). Again, epinephrine infusion produced a similar increase in hindlimb blood flow but did not alter laser Doppler signal. These data (like the 1-MX result) could be explained on the basis of insulin-induced microvascular recruitment.

Most recently, we have used ultrasound during albumin microbubble infusion to estimate microvascular volume (MV) in rat skeletal muscle in vivo (11). Using contrast-enhanced ultrasound (CEU), we observed that exercise, a known stimulus for microvascular recruitment in muscle, enhanced MV. This technique is well suited to repeated measurement of microvascular perfusion in small animals and humans.

In the current study, we measured 1-MX metabolism and imaged microvascular perfusion (using CEU) in rat hindlimb muscle in vivo to ascertain whether physiological hyperinsulinemia exerts a hemodynamic action to increase microvascular perfusion. We then used CEU to examine whether insulin-induced changes in microvascular recruitment and total limb blood flow are temporally coupled.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing 250–350 g were obtained from Hilltop Laboratory Animals (Scottsdale, PA). Animals were housed at $22 \pm 2^\circ\text{C}$ and

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β , microvascular flow velocity; CEU, contrast-enhanced ultrasound; 1-MU, 1-methylurate; MV, microvascular volume; 1-MX, 1-methylxanthine.

during a 12:12 h light:dark cycle. The animals were provided food and water ad libitum; however, food was removed the night before the experiment. All experiments were approved by the Animal Care and Use Committee of the University of Virginia.

Surgery. The hyperinsulinemic clamp was used as described previously (8). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (55 mg/kg body wt). Polyethylene (PE50; Intramedic) cannulas were inserted into the carotid artery for arterial blood sampling and measurement of arterial blood pressure (Transpac IV pressure transducer; Abbott Critical Systems) and into both jugular veins for intravenous infusions. A tracheotomy tube was placed, and the animals breathed spontaneously throughout the experiment. The femoral vessels in the left leg were exposed by an ~1.5-cm incision through the skin overlaying the vessels. The femoral artery was carefully separated from the femoral vein and saphenous nerve. The epigastric vessels were ligated, and a time transit flow probe that measured blood flow continuously by ultrasound (VB series 0.5 mm; Transonic Systems) was positioned over the femoral artery. The flow probe was interfaced through a flow meter to an IBM-compatible computer. Femoral blood flow, arterial blood pressure, and heart rate were continually measured using Windaq data acquisition software (Dataq Instruments). The animal was maintained under anesthesia for the duration of the experiment with aqueous sodium pentobarbital ($0.6 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) via the carotid artery. A heat lamp positioned above the rat maintained the rat's body temperature.

Experimental protocols. After a 60-min stabilization period following completion of the surgical procedure, rats were divided into two groups. Group 1 animals received either normal saline ($10 \mu\text{l}/\text{min}$, $n = 6$) or insulin ($3.0 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $n = 9$) intravenously while maintaining blood glucose at baseline via a variable-rate infusion of 30% dextrose.

For the current study, MV and flow velocity were assessed with CEU at baseline and after 120 min of infusion. In addition, 1-MX metabolism was determined at the end of the 120-min infusion. In group 2 animals ($n = 5$), the time course of insulin's action on microvascular blood volume and velocity were assessed by CEU. Measurements were performed at baseline and at 30 and 90 min after initiating insulin infusion. These time points were selected to reflect times at which plasma insulin concentration increases most markedly after a meal (12,13).

Glucose (30% wt/vol solution) was infused at a variable rate into all insulin-treated animals to maintain euglycemia, and arterial blood glucose was monitored every 10 min for the first hour and then every 15 min for the rest of the study, using an Accu-Chek blood glucose monitoring system (Roche, Indianapolis, IN).

CEU. CEU has been used extensively in the past to measure microvascular flow in the myocardium (14–16). Estimates of microvascular flow using CEU have been validated using 11- μm radiolabeled microspheres to show that there is a strong correlation between the two techniques ($r = 0.96$, $P < 0.001$) (14). The technique was modified for its use for the rat as follows: a linear-array transducer interfaced with an ultrasound system (HDI-5000; ATL Ultrasound) was positioned over the right leg of the rat and secured for the course of the experiment. The adductor magnus and semimembranosus muscles of the hindlimb were imaged in short-axis with intermittent harmonic imaging at a transmission frequency of 3.3 MHz. The mechanical index [peak negative acoustic pressure] \times [frequency]^{-1/2}, a measure of acoustic power, was set at 0.9. The acoustic focus was set at the mid-portion of the muscle group. Gain settings were optimized and held constant. Data were recorded on 1.25-cm videotape using a S-VHS recorder (Panasonic MD830; Matsushita Electric). Albumin microbubbles containing octafluoropropane gas (Optison; Mallinckrodt Medical) were infused intravenously at $120 \mu\text{l}/\text{min}$ for the duration of data acquisition. The acoustic signal that is generated from the microbubbles exposed to ultrasound is proportional to the concentration of microbubbles within the volume of tissue being imaged. Essentially, all microbubbles within the ultrasound beam are simultaneously imaged and destroyed in response to a single pulse of high-energy ultrasound (14). As the time between successive pulses is prolonged, the beam becomes progressively replenished with microbubbles (14). Eventually, the beam will be fully replenished, and further increases in the time between each pulse will not affect microbubble signals in tissue (Fig. 1). The rate of microbubble reappearance within the ultrasound beam provides an indication of microvascular flow velocity (β), and the plateau video intensity reached at long pulsing intervals provides a measurement of MV (14).

For the current study, images were acquired during continuous imaging and at pulsing intervals from 1 to 20 s. Image analysis was performed off-line. Frames were aligned by cross-correlating several frames at each pulsing interval and were separately averaged and digitally subtracted from averaged frames obtained during continuous imaging (obtained at a pulsing interval of 30 ms), which served as background. Using continuous imaging frames as background allowed for the elimination of the contribution by larger vessels

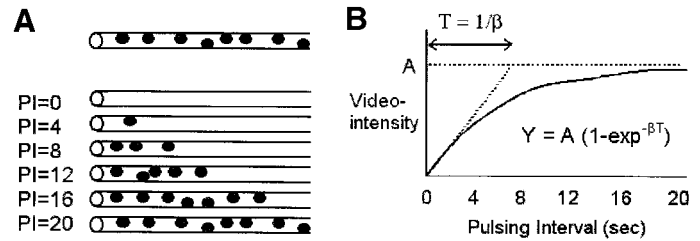


FIG. 1. A: Schematic depiction of the replenishment of microbubbles in capillaries after a pulse of high-energy ultrasound. As the time between each ultrasound pulse is prolonged, more microbubbles reappear. PI, pulsing interval. **B:** A typical relation between pulsing interval and acoustic signal, measured as video intensity. As the pulsing interval is prolonged, the number of microbubbles within the capillaries increases, resulting in a higher video intensity. Eventually a plateau is reached where the time interval between each pulsing interval does not cause a further increase in video intensity due to complete replenishment of the vessels in the beam. The asymptote that intercepts the y -axis is the maximal video intensity signal and a measurement of MV. The x -intercept of the y -axis asymptote and the tangent to the upward sloping hyperbolic function is a measure of the rate constant of video intensity rise (β), an indicator of microvascular flow velocity.

(velocity $>100 \mu\text{m}/\text{s}$) to overall video intensity. The background-subtracted video intensity at each pulsing interval was measured within the area of interest in muscle, and pulsing interval versus video intensity data were fitted to the function [$y = A(1 - e^{-\beta t})$], where y is video intensity, t is the pulsing interval, A is plateau video intensity (an index of MV), and β is the rate constant, which provides a measure of microvascular flow velocity (Fig. 1) (14).

To determine the appropriate microbubble infusion dose for in vivo imaging and to ensure linearity in the microbubble concentration versus video intensity relation, we performed experiments to define a microbubble dose-response curve both in vivo and in vitro. For the in vitro experiment, the ultrasound probe was immersed in a beaker of water, and video intensity was measured after injection of incremental numbers of microbubbles during continuous mixing with a stir bar. For the in vivo experiment, microbubbles were infused into the jugular vein of the rat, and the ultrasound probe was positioned over the thigh of the rat as described above. Background-subtracted video intensity in resting skeletal muscle was determined during step-wise increases in microbubble concentration. A microbubble infusion rate of $120 \mu\text{l}/\text{min}$ was selected for the in vivo study because this dose resulted in video intensity measurements that were well within the linear range of the microbubble concentration and video intensity indicated by both the in vitro and in vivo dose-range experiments, thus making it possible to accurately detect an increase in MV.

1-MX metabolism. To date, the best indicator that we have that 1-MX metabolism reflects microvascular perfusion comes from studies of the effect of electrical stimulation of muscle (11,17). We have shown that 1-MX metabolism increases in response to electrical stimulation (a known stimulus for capillary recruitment) in rat hindlimb both in vivo (11) and in a perfused preparation (17). In the perfused rat hindlimb preparation, total blood flow is held constant (17), yet 1-MX conversion to 1-MU is increased, suggesting that microvascular perfusion can be affected independently of total flow.

In group 1 rats, 1-MX ($0.4 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) was infused intravenously at a constant rate during the last 60 min of each study. It was shown in previous experiments (8) that 1-MX is rapidly metabolized to 1-MU and that it is necessary to partially inhibit the xanthine oxidase before infusing 1-MX to slow its whole-body clearance and to allow for measurable concentrations in arterial and venous blood. We have performed allopurinol dose-response curves in the rat in vivo (data not shown) and found that $10 \mu\text{mol}/\text{kg}$ partially inhibited the xanthine oxidase and allowed steady-state systemic levels of 1-MX to be obtained.

At the end of each experiment in rats from group 1, blood from the carotid artery and femoral vein was sampled and immediately centrifuged, and $100 \mu\text{l}$ of plasma was added to $20 \mu\text{l}$ of 2 mol/l perchloric acid. The plasma was immediately neutralized with $12 \mu\text{l}$ of 2.5 mol/l K_2CO_3 and stored at -20°C until analysis of 1-MX.

Plasma 1-MX, 1-MU, allopurinol, and oxypurinol were analyzed using reverse-phase high-performance liquid chromatography from a modified version of the protocol used by Wynants et al. (18). The metabolites were separated and analyzed using an Ultrasphere ODS column (25 cm, 5-mm particles; Beckman) at $1.0 \text{ ml}/\text{min}$. Two buffers were used for the aqueous mobile phase. Buffer A consisted of 4.35 mmol/l acetic acid, and buffer B

TABLE 1

Effect of saline and insulin on systemic and hindleg hemodynamic factors, blood glucose concentrations, and glucose infusion rate required to maintain basal blood glucose at the end of a 120-min infusion period

	Saline	Insulin
Mean arterial pressure (mmHg)	105 ± 5	99 ± 5
Heart rate (beats/min)	294 ± 21	309 ± 22
Hindlimb vascular resistance (mmHg · min · ml ⁻¹)	168 ± 20	106 ± 12*
Arterial blood glucose (mg/dl)	84 ± 7	82 ± 8
Final glucose infusion rate (mg · min ⁻¹ · kg ⁻¹)	—	11.3 ± 2.4

Data are means ± SE; *n* = 6 for the saline group and *n* = 8 for the insulin group. **P* < 0.05 compared with saline, Student's unpaired *t* test.

consisted of 87 mmol/l acetic acid/20% methanol/5% acetonitrile/0.2% tetrahydrofuran, pH 4.0. Buffer A was used during 0–10 min, and at that time buffer B was introduced and buffer A was decreased by using a linear gradient from 10 to 30 min. Buffer B was used from 30 to 40 min. Detection between 0 and 20.5 min was at a wavelength of 250 nm, and between 20.5 and 40 min it was at 272 nm.

Statistical analysis. For animals studied in group 1, an unpaired Student's *t* test was used to test differences between saline- and insulin-infused rats for mean arterial blood pressure, heart rate, arterial blood glucose level, vascular resistance, and 1-MX metabolism after 120 min of infusion. A paired Student's *t* test was used to compare the effects of insulin or saline versus baseline on MV, β, and femoral artery blood flow after 120 min of infusion. For group 2, where each animal served as its own control, repeated-measures analysis of variance was used to test differences between baseline and 30 and 90 min of insulin infusion on MV, β, and femoral artery flow. When a significant difference was found, pairwise comparisons by Student-Newman-Keuls test were used to determine at which individual times the differences were significant. For all comparisons, significance was recognized at *P* < 0.05.

RESULTS

The effect of insulin on microvascular recruitment and femoral artery flow. In rats in group 1, arterial blood glucose concentrations remained constant throughout the experiment and were similar in insulin- and saline-treated animals (Table 1). To maintain euglycemia, the insulin group required glucose infusion at an average rate of 11.3 ± 2.4 mg · min⁻¹ · kg⁻¹. Insulin infusion increased the plasma insulin concentration from 52 ± 13 to 599 ± 58 pmol/l (*P* < 0.05, *n* = 4). Compared with saline, insulin infusion for 120 min did not alter mean arterial pressure or heart rate (Table 1). However, hindleg vascular resistance (mean arterial pressure/femoral artery flow) declined significantly (*P* < 0.05) in response to insulin, and femoral artery flow increased (0.64 ± 0.05 to 1.02 ± 0.10 ml/min; *P* < 0.05) with insulin but not with saline (0.79 ± 0.13 to 0.68 ± 0.09 ml/min) (Fig. 2 and Table 2). Table 2 shows that femoral artery flow was significantly higher after 120 min of insulin infusion compared with saline (*P* < 0.05).

Compared with baseline, MV markedly increased with insulin (167.0 ± 39.8%) (Table 2) at 120 min. This change was significantly greater (*P* < 0.05) than the 28.2 ± 13.8% increase seen with saline (Table 2). In response to insulin or saline, β did not significantly change from baseline (Table 2).

The effect of insulin and saline on arterial 1-MX concentrations and hindleg 1-MX metabolism is shown in Fig. 3. At the end of the 120-min experimental period, arterial 1-MX concentration was similar between saline and insulin (18.3 ± 3.6 vs. 16.8 ± 2.7 μmol/l). Hindleg 1-MX extraction

during saline infusion was 7.1 ± 0.8 μmol/l, and this was not significantly different from the insulin-treated group (7.7 ± 1.2 μmol/l). However, because of differences in total blood flow, 1-MX metabolism was 47% higher (*P* < 0.01) for insulin than saline, consistent with the conclusion that insulin had enhanced the exposure of 1-MX to xanthine oxidase by recruiting capillaries.

Time course of insulin's action on microvascular flow. Figure 4A shows the background-subtracted color-coded CEU images (pulsing interval of 20 s) at baseline and after 30 and 90 min of insulin infusion. Progression of the color from red to orange to yellow signifies enhanced video intensity. Insulin produced a higher peak video intensity (MV) at this pulsing interval at both 30 and 90 min of infusion compared with baseline. Figure 4B shows the corresponding pulsing interval (1–20 s) versus video intensity data at baseline and after 30 and 90 min of insulin infusion. Again, insulin at 30 min produced a higher plateau video intensity (27 units) than baseline (17 units), indicating increased MV. β did not change. The pulsing interval versus video intensity curve obtained at 30 min was similar to that seen at 90 min of insulin infusion.

Table 3 shows the effect of insulin on MV, β, and femoral artery flow at baseline and at 30 and 90 min of infusion in

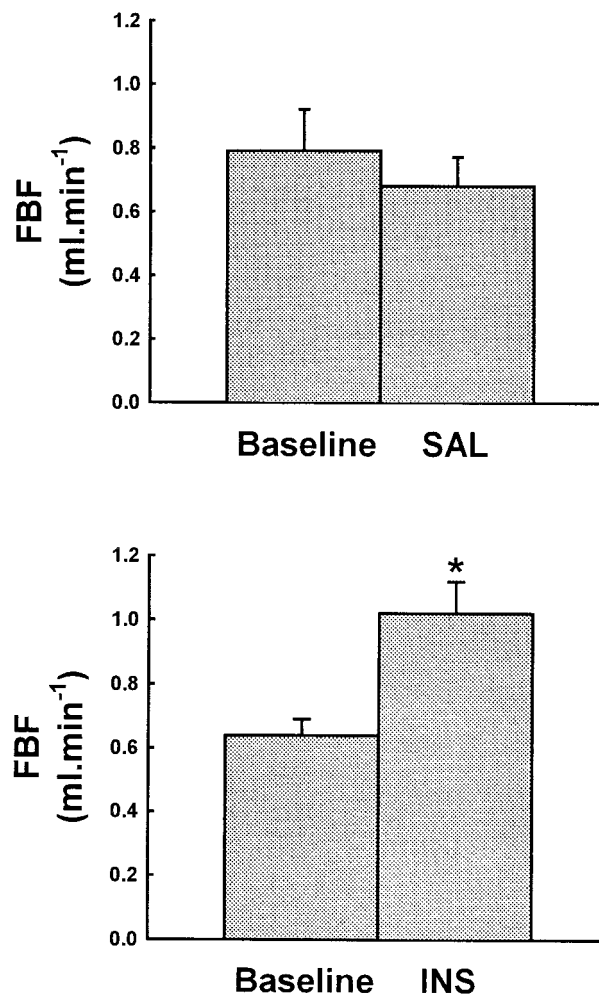


FIG. 2. Femoral artery blood flow in response to saline (SAL) or insulin (INS) at the end of the 120-min infusion period. Values are the means ± SE for six (saline) and nine (insulin) animals. *Significantly different (*P* < 0.05) from baseline.

TABLE 2

Effect of saline and insulin infusion for 120 min on femoral artery blood flow measured by flow probe, MV, and β measured by CEU

	Baseline	Saline	Baseline	Insulin	<i>P</i> (saline vs. insulin at 120 min)
FBF (ml/min)	0.79 ± 0.13	0.68 ± 0.09	0.64 ± 0.05	1.02 ± 0.10*	<0.05
Δ FBF (%)	—	-11.8 ± 6.6	—	62.7 ± 14.9	<0.005
MV (video intensity)	6.1 ± 0.9	7.5 ± 0.7	6.7 ± 1.7	13.4 ± 1.8†	<0.05
Δ MV (%)	—	28.2 ± 13.8	—	167.0 ± 39.8	<0.05
β (s ⁻¹)	0.10 ± 0.03	0.09 ± 0.02	0.11 ± 0.02	0.14 ± 0.02	NS
Δ β (%)	—	15.0 ± 34.3	—	29.5 ± 13.9	NS

Data are means ± SE; *n* = 6 for the saline group and *n* = 9 for the insulin group. *P* value compares saline to insulin after 120 min (Student's unpaired *t* test). **P* < 0.05 and †*P* < 0.001 compared with baseline (Student's paired *t* test). FBF, femoral artery blood flow.

the second group of animals. MV was significantly enhanced by 30 min (12.9 ± 1.8 to 18.9 ± 1.8; *P* < 0.001) of insulin infusion, and this was of similar magnitude to the changes observed at 90 min (20.6 ± 2.2; *P* < 0.001). This occurred at times when there was no discernable change in femoral artery flow. We estimated that we have a >80% power to detect a 20% change in blood flow within 30 min of insulin infusion. β at these times did not differ from baseline. However, the percent increase in MV appeared to be higher at 120 min in the first group (Table 2) than at the shorter times in the second group (Table 3). The difference in baseline MV between the two experimental groups is due at least in part to two different batches of microbubbles and modest changes in the gain settings. However, because we measured MV and β at baseline and during the insulin or saline infusion, each animal served as its own control.

DISCUSSION

We have previously used two approaches to examine the microvascular action of insulin in rat muscle in vivo. One, a biochemical approach that measures hindlimb 1-MX metabolism (8), and the second, laser Doppler flowmetry (10), each suggested that pharmacological concentrations of insulin enhance microvascular recruitment in rat muscle in vivo. In the current study, we examined the action of physiological increases in insulin to recruit microvascular vessels, using two independent methods. Physiological hyperinsulinemia significantly increased 1-MX metabolism to a degree similar to that previously seen with higher doses of insulin (8). Using CEU, we again saw that insulin increased microvascular blood volume, consistent with microvascular recruitment within muscle. The concordant results observed using two very different approaches suggest that the methods are comparable for assessing microvascular flow, and they provide strong evidence for a physiological action of insulin to regulate microvascular perfusion.

The relative changes in 1-MX metabolism and CEU were not identical; indeed, within the CEU, results seen in groups 1 and 2 were not quantitatively identical. There are a number of things that might contribute to both the 1-MX and CEU measurements and thus influence the quantitative nature of the responses. First, the 1-MX measurement is made across the whole hindlimb (arteriovenous difference × flow), whereas the CEU measurement is made in one area and exclusively in muscle. Therefore, if insulin had no effect to increase flow in nonmuscle tissue (bone and skin), the proportionate rise in MV based on the CEU

measurement would be greater than for the 1-MX measurement. Likewise, it is known that there are significant differences in insulin-induced increases in microsphere perfusion among different types of muscle (i.e., red versus white fibers). Beyond that, it should be recognized that 1-MX metabolism and CEU are fundamentally different techniques for estimating microvascular recruitment. Microbubbles are entirely intravascular and produce a signal that we can measure as video intensity. The background subtraction procedure that we use allows us to remove the contribution from larger vessels, in which flow is rapid; this cannot be done with 1-MX, although it is thought that xanthine oxidase is predominantly a microvascular endothelial marker.

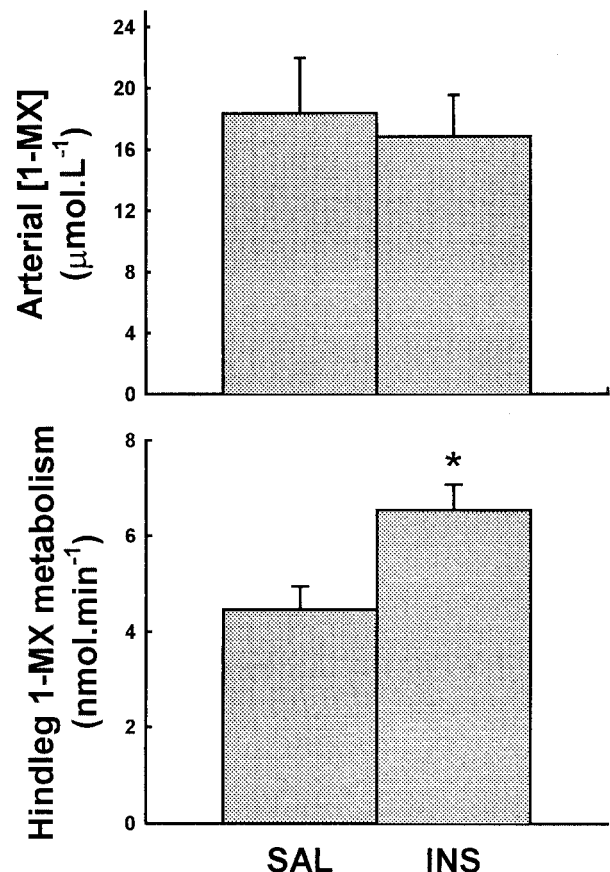


FIG. 3. Systemic and hindleg 1-MX values of saline (SAL) and insulin (INS) at the end of the 120-min infusion period. Values are means ± SE for six and five animals, respectively. *Significantly different (*P* < 0.01) from saline value.

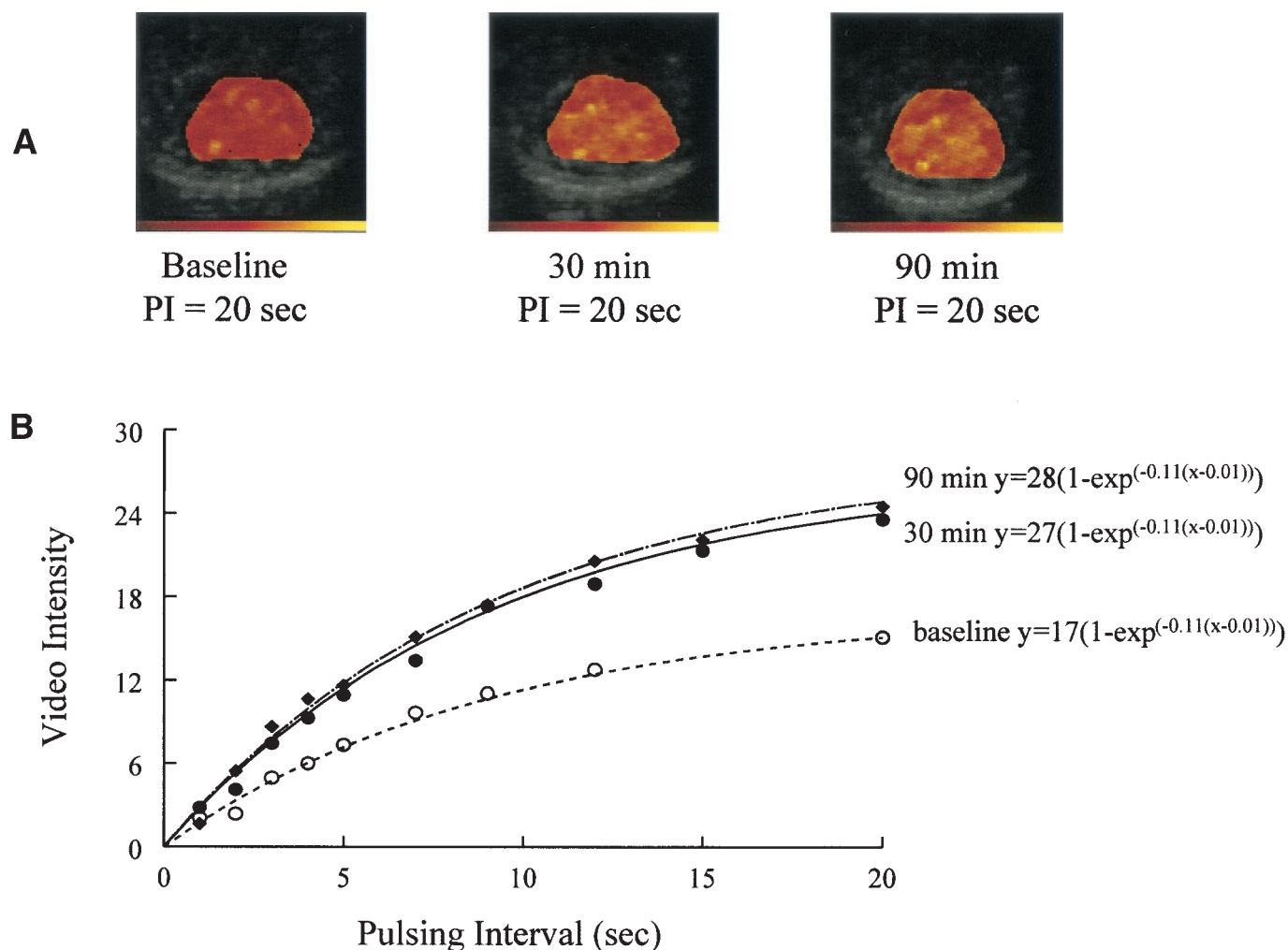


FIG. 4. **A:** Examples of background-subtracted color-coded contrast ultrasound images observed at a pulsing interval of 20 s during baseline and after 30 and 90 min of insulin infusion. Color changes from red to orange to yellow represent increasing MV. Color scale (256 levels) for background-subtracted video intensity is at the bottom of each image. **B:** Typical trace of pulsing interval versus video intensity during baseline and after 30 and 90 min of insulin infusion. Data are obtained from adductor brevis and semimembranosus muscles of the rat.

Inasmuch as CEU is a noninvasive method, it permitted repeated measurement of insulin's vascular action in individual animals. We observed that insulin increased MV well before it affected hindlimb total blood flow. Indeed, the changes in steady-state video intensity (MV) at 30 and 90 min during the insulin infusion was not different from those seen at 120 min, by which time total hindlimb flow had begun to rise. This suggests that microvascular recruitment occurred early and remained stable for the duration of the infusion period, and that it occurred independently of changes in total flow. Therefore, insulin-mediated microvascular recruitment and total limb flow follow a different time course. This result is reminiscent of our previous findings using pharmacological doses of insulin ($10 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$). In that study, insulin infusion caused an increase in laser Doppler signal in muscle within 20 min that reached a maximum by 50 min. Femoral artery flow measured continuously for the duration of the experiment was not increased until at least the 60 min time point (10). Thus, increases in laser Doppler signal preceded by 30–40 min any changes in total muscle blood flow. The marked increase in femoral artery flow seen at 60 min in the laser Doppler study was likely caused

by the higher dose of insulin ($10 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) used for the former study. As noted earlier, we have also reported that epinephrine infusion increases rat hindlimb blood flow without recruiting capillaries, measured by either hindleg 1-MX metabolism or laser Doppler flowmetry (8,10), again providing experimental evidence that changes in microvascular flow can be dissociated from

TABLE 3

Time course of insulin action on femoral artery blood flow measured by flow probe, MV, and β measured by CEU

	Baseline 0 min	Insulin 30 min	Insulin 90 min
FBF (ml/min)	0.58 ± 0.08	0.55 ± 0.05	0.65 ± 0.08
Δ FBF (%)	—	4.0 ± 3.7	13.8 ± 9.4
MV (video intensity)	12.9 ± 1.8	$18.9 \pm 1.8^*$	$20.6 \pm 2.2^*$
Δ MV (%)	—	52.8 ± 14.8	64.6 ± 9.9
β (s^{-1})	0.11 ± 0.03	0.10 ± 0.02	0.11 ± 0.02
$\Delta \beta$ (%)	—	-2.2 ± 10.2	8.6 ± 15.2

Data are means \pm SE; $n = 5$ for each group. $*P < 0.001$ compared with baseline, repeated measures ANOVA, post hoc Student-Newman-Keuls test.

changes in total limb flow. Kuznetsova et al. (19) have also shown dissociation between total muscle flow and microvascular flow, as measured by microspheres and laser Doppler flowmetry, respectively. In those studies, angiotensin II or phenylephrine infusion into the anesthetized rat during ganglionic blockade resulted in an increase in laser Doppler signal but no change in total flow. In contrast, isoproterenol markedly enhanced total muscle flow, despite a reduction in laser Doppler signal.

In addition to microvascular blood volume, CEU provides an estimate of β . The current results showed that β did not change significantly during the first 90 min of insulin infusion but may have increased at 120 min, a time when hindlimb flow had likewise risen. This is not surprising because the product of $\beta \times MV$ should provide an index of flow rate at a microvascular level. However, because this product increased without accompanying changes in femoral artery blood flow at 30 and 90 min of insulin infusion, the possibility should be considered that in addition to microvascular recruitment, insulin redistributed flow at the microvascular level within the rat hindleg. Femoral artery blood flow is a measurement of total hindleg flow that feeds muscle, bone, skin, and fat, whereas our measurement of MV and β is determined within a discrete area of the hindleg that is exclusively in muscle. Thus, it is possible to find a mismatch between total hindleg blood flow and $MV \times \beta$ within this small area of muscle. For example, we have found in the constant-flow perfused rat hindlimb that microvascular recruitment can increase in muscle without any change in total hindleg flow, and that this is most likely caused by flow redistribution within the hindlimb. Also, as noted previously, muscle blood flow in the rat hindlimb is very heterogeneous, based on the distribution of the different fiber types (20). For instance, in the anesthetized rat, blood flow (measured by 15- μm microspheres) to the red quadriceps is $9 \text{ ml} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$ but is only $4 \text{ ml} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$ in the white quadriceps and the extensor digitorum longus. Thus, based on the distinct differences in total muscle blood flow for each individual muscle of the rat hindlimb, it is highly likely that there could be a mismatch between total hindlimb flow and microvascular flow within a discrete area of muscle.

The albumin microbubbles used as the contrast-enhancing agent averaged 4 μm in diameter, had similar rheology to red blood cells, and were contained entirely within the vascular space (21). As a result, they were an excellent perfusion tracer. Furthermore, over the range of video intensity studied here, there was a linear correlation between the concentration of microbubbles within the vasculature and video intensity. CEU is particularly useful because it allows background subtraction of the tissue image and thus elimination of signal from large vessels, which fill very rapidly, allowing microvascular flow (which is slower) to be imaged selectively. The CEU method has the additional appeal that it allows imaging of specific regions within muscle and, unlike 1-MX metabolism or measures of total hindlimb flow, is not influenced by contributions from nonmuscle tissue (skin, bone, and adipose tissue). Furthermore, compared with laser Doppler flowmetry, CEU samples a relatively large volume of muscle and does not require surgical exposure of the

muscle surface. The laser Doppler method applied to muscle has the potential for producing artifacts, given that it only samples the immediate region around an implanted or surface probe.

Several other investigators have attempted to determine the effect of insulin on muscle microvascular flow. Raitakari et al. (22) used positron emission tomography during inhalation of [^{15}O]carbon monoxide. The labeled carbon monoxide binds to erythrocytes, thereby providing a vascular tracer. High-dose insulin concentrations augmented leg blood flow and increased muscle blood volume by 9%. The high dose of insulin used limited any effort to separately assess effects on total flow from those on microvascular recruitment. Moreover, the positron emission tomography method measures blood within both the larger vessels and the microvasculature; thus, changes in MV may not be accurately quantified.

In a subsequent study, Bonadonna et al. (23) used a very different approach and injected a bolus of nonmetabolizable radiolabeled L-glucose into the brachial artery to measure forearm transit time and to estimate extracellular volume. Pharmacological concentrations of insulin enhanced total forearm blood flow, as measured by the dilution of intra-arterially infused indocyanine green dye. The mean transit time of L-glucose lengthened in response to insulin because of an expansion of the extracellular volume by 33%. This measure includes more than the vascular space, and would not report separately on large and small vessels.

More recently, Baron et al. (24) modeled the effects of insulin on microvascular flow by raising and lowering blood flow in the human leg during a steady-state insulin clamp and compared the results to the predicted values of the noncapillary recruitment model of Renkin (25). It was concluded that because glucose uptake was greater than predicted when leg blood flow was increased and smaller than predicted when leg blood flow was decreased, this suggested that the number of perfused capillaries changed considerably under insulin-stimulated conditions, assuming that glucose permeability remained constant. Although it was concluded that insulin-mediated increments in muscle perfusion are accompanied by microvascular recruitment, no direct experimental measure of microvascular recruitment was made.

The relation between insulin's action on total muscle blood flow and its metabolic actions remains embedded in controversy. Yki-Jarvinen and Utriainen (2) have concluded that insulin's effect on total limb flow occurs after its metabolic effects on glucose uptake. It should be noted that this temporal discordance is based on the assumption that insulin's vascular action is restricted to increases in total blood flow. The present study indicated that insulin produces an early hemodynamic action at the microvascular level during a time that insulin is producing a metabolic action. Interestingly, microvascular recruitment (as measured by laser Doppler flowmetry) closely follows changes in glucose infusion rate and not total muscle flow (10). This relation also held for the current study because the glucose infusion rate at 30 min of insulin infusion was 90% of the glucose infusion rate used at 90 min (the plateau for insulin's metabolic action).

Insulin-stimulated total blood flow in many insulin-

resistant disease states, such as type 2 diabetes, hypertension, and obesity, is reported to be blunted (1,4,26,27), providing further evidence that the hemodynamic involvement of insulin may be important for its action. However, controversies regarding the vascular actions of physiological doses of insulin persist because some investigators have failed to see an increase in blood flow within healthy humans (28) or an impairment during insulin-resistant states (29,30). None of these studies address separately the actions of insulin on microvascular recruitment and total flow. This is unfortunate because, based on the theoretical models of tissue perfusion developed by Renkin (25), microvascular recruitment might be expected to be a more potent determinant of tissue glucose exchange than total flow. For instance, we have recently reported that when insulin-mediated microvascular recruitment is prevented with the vasoconstrictor α -methyl-5-HT, an acute insulin-resistant state is induced (31).

In summary, these results indicate that insulin at physiological concentrations recruits flow to the microvasculature in muscle, and at the insulin concentration used here, this effect precedes by at least 60 min any changes in total muscle blood flow. Changes in total muscle blood flow have later onset and do not involve further changes in the microvasculature. Therefore, a full assessment of the role of insulin's vascular actions in healthy and insulin-resistant states may require focusing on the effects on both total blood flow and microvascular flow, which could be the key to insulin's vascular action.

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