Microvascular Recruitment Is an Early Insulin Effect That Regulates Skeletal Muscle Glucose Uptake In Vivo

Michelle A. Vincent,[1] Lucy H. Clerk,[1] Jonathan R. Lindner,[1] Alexander L. Klibanov,[1] Michael G. Clark,[2] Stephen Rattigan,[2] and Eugene J. Barrett[1]

Insulin increases glucose disposal into muscle. In addition, in vivo insulin elicits distinct nitric oxide synthase–dependent vascular responses to increase total skeletal muscle blood flow and to recruit muscle capillaries (by relaxing resistance and terminal arterioles, respectively). In the current study, we compared the temporal sequence of vascular and metabolic responses to a 30-min physiological infusion of insulin ($3 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, euglycemic clamp) or saline in rat skeletal muscle in vivo. We used contrast-enhanced ultrasound to continuously quantify microvascular volume. Insulin recruited microvasculature within 5–10 min ($P < 0.05$), and this preceded both activation of insulin-signaling pathways and increases in glucose disposal in muscle, as well as changes in total leg blood flow. Moreover, L-NAME (N^{ω}-nitro-L-arginine-methyl ester), a specific inhibitor of nitric oxide synthase, blocked this early microvascular recruitment ($P < 0.05$) and at least partially inhibited early increases in muscle glucose uptake ($P < 0.05$). We conclude that insulin rapidly recruits skeletal muscle capillaries in vivo by a nitric oxide–dependent action, and the increase in capillary recruitment may contribute to the subsequent glucose uptake. *Diabetes* **53:1418–1423, 2004**

Insulin enhances both skeletal muscle glucose disposal and total leg blood flow in a time- and dose-dependent fashion (1,2). It has been proposed that increasing blood flow by this action on resistance vessels could facilitate the delivery of glucose and insulin and thereby contribute to overall glucose disposal (1). However, the observations that in most, but not all, studies insulin-induced increases in total muscle blood flow temporally lag significantly behind the stimulation of muscle glucose uptake (2) and that this flow effect requires relatively high insulin concentrations have led to controversy regarding the physiological significance of insulin-induced flow increases (2,3). The observation that

inhibition of nitric oxide (NO) synthase by N^{ω}-mono-methyl-L-arginine (L-NMMA) abolishes insulin-induced increases of leg blood flow in humans and diminishes (~25%) glucose uptake (4) supports a physiological role for insulin action on the vasculature. However, within muscle, nitric oxide synthase (NOS) is present in both myocytes (μ-NOS, a variant of the neuronal NOS [nNOS] isoform) (5,6) and endothelium (eNOS) (7), and the locus of the NO production that mediates insulin-dependent increases of total muscle blood flow and glucose uptake is unknown. The observation that eNOS-deficient mice are hypertensive and resistant to insulin-mediated increases of muscle glucose uptake suggests a role for eNOS (8,9); however, nNOS-deficient mice are also insulin resistant (8). Observations that endothelial cells have insulin receptors and that insulin stimulates phosphorylation and activation of protein kinase B (Akt), which can then phosphorylate and activate eNOS (10–12), support a direct action of insulin on the endothelium.

The recent report that endothelial cell insulin receptor null mice show no major abnormality in vascular development or glucose homeostasis may be evidence against an important role for vascular recruitment in regulating glucose disposal (13). However, those animals are insulin resistant when placed on a low-salt diet and show evidence of altered expression of both eNOS and endothelin-1 that may play a role in maintaining insulin sensitivity.

We recently reported, using three independent methods for assessing microvascular recruitment (14–17), that insulin provokes microvascular recruitment in skeletal muscle. Using contrast-enhanced ultrasound (CEU), we observed that while insulin required 120 min to augment rat hindlimb blood flow, it increased microvascular volume in skeletal muscle within 30 min, and this effect persisted throughout the insulin infusion (17), implying that small terminal arterioles respond more promptly to insulin than the resistance vessels (18). Whether this arises as a direct vascular response to insulin or indirectly from insulin-induced effects on muscle metabolic function is not known.

In the current study, we examined whether insulin-mediated capillary recruitment precedes activation of insulin signaling and augmented glucose metabolism within muscle tissue in vivo. In addition, we reasoned that if an early microvascular effect of insulin is important for glucose disposal, then NOS inhibition would diminish even the early increments of glucose uptake that precede any insulin-induced increases in total limb blood flow.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing 250–350 g (Hilltop Laboratory Animals, Scotdale, PA) were housed at 22 ± 2°C and maintained with a 12-h/12-h light/dark cycle. Animals were provided with food and water ad libitum and then fasted overnight before study. The University of Virginia Animal Care and Use Committee approved the experimental protocols. Rats were anesthetized with sodium pentobarbital (55 mg/kg body wt i.p.), and polyethylene cannulae were inserted into the carotid artery for blood sampling and measurement of blood pressure and into both jugular veins for intravenous infusions. A tracheostomy was performed to facilitate spontaneous respiration during the experiment. Both femoral vessels were exposed via a 1.5-cm incision, and the left femoral artery was carefully separated from the femoral vein and nerve, the epigastric vessels were ligated, and a flow probe (Transonic Systems, VB series 0.5 mm) was positioned over the artery. The probe was interfaced through a flow meter to an IBM-compatible computer. Femoral artery blood flow, blood pressure, and heart rate were measured continuously using WINDAQ data acquisition software (DATAQ Instruments). Anesthesia was maintained by a continuous infusion of aqueous sodium pentobarbital (0.6 mg · min^{-1} · kg^{-1}) via the carotid artery. Rats were then maintained for a 1-h basal stabilization period before beginning each of three infusion protocols.

Infusion protocols. After a 1-h basal period, two groups of animals were studied using each of three infusion protocols. The first protocol involved infusion of saline only, the second involved regular insulin (6 mU · min^{-1} · kg^{-1} × 5 min, then 3 mU · min^{-1} · kg^{-1} × 25 min, euglycemic insulin clamp), and the third involved the same insulin infusion with a superimposed infusion of the NOS inhibitor N^ω-L-nitro-arginine-methyl ester (L-NAME) (50 µg · min^{-1} · kg^{-1}). The infusion rate for L-NAME was selected after preliminary experiments demonstrated that it caused an ~20-mmHg rise in mean arterial pressure, indicating effective antagonism of NOS (18). The L-NAME infusion was begun with the 30-min basal period and persisted throughout insulin infusion. The volume of saline infused during protocol 1 was comparable to that used during insulin/glucose infusion in the other two protocols. In one of the two groups of animals studied with each of these three infusion protocols, we measured microvascular blood volume (CEU) and glucose metabolism (glucose infusion rate to maintain euglycemia) in vivo at baseline and serially during a 30-min experimental period. In the second group, we positioned an ultrasonic flow probe around the femoral artery (Transonic Systems, Ithaca, NY) to measure total limb blood flow and measured arterial-venous glucose differences at 0, 7, 15, and 30 min. Calf muscle samples were obtained at 0, 7, and 30 min to measure phosphorylation of Akt. Two separate groups were required for each protocol because Doppler measurements of femoral blood flow cannot be obtained during microbubble infusion. Four to six rats were studied in each protocol in both groups.

CEU measurements. Using CEU imaging, we measured microvascular blood volume continuously throughout each infusion. This technique relies on the ultrasound detection of a microbubble contrast agent composed of a lipid shell and a perfluorocarbon gas core. These microbubbles average 4 µm in diameter, remain entirely within the vascular space, and have similar rheology to erythrocytes (19). As a result, they are an excellent perfusion tracer. CEU allows imaging of specific regions within muscle and is not influenced by contributions from nonmuscle tissue (skin, bone, and adipose). 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 (20,21). As the time between successive pulses is prolonged, the beam becomes progressively replenished with microbubbles. Eventually, the beam will be fully replenished and further increases in the time between each pulse will not affect the microbubble signal in tissue. The rate of microbubble reappearance within the ultrasound beam provides an indication of microvascular flow velocity, and the plateau video intensity reached at long pulsing intervals provides a measurement of microvascular volume, a measure of microvascular recruitment (22). In addition, by using background subtraction, the signal from the tissue as well as from larger vessels (which fill rapidly) can be eliminated. We have previously reported that the time required to reach plateau video intensity is ~10 s in rat muscle (23).

To acquire images, a linear array, a transducer interfaced with an ultrasound system (HDI-5000; Philips Medical Systems, Andover, MA), was positioned over the right hindlimb of the rat to image the proximal adductor muscle group (adductor magnus and semi-membranous) and was secured for the duration of the experiment. Pulse inversion imaging was performed at a transmission frequency of 3.3 MHz and a mechanical index (peak negative acoustic pressure divided by the square root of the frequency) of 0.8. The acoustic focus was set at the mid-portion of the muscles. Gain settings were optimized and held constant. Data were recorded on a 1.25-cm videotape using an S-VHS recorder. Phospholipid-coated perfluorocarbon gas micro-

bubbles were manufactured at the University of Virginia as reported previously (24). Microbubble concentration and size distribution were determined by electrozone sensing with a Coulter Multisizer IIe (Beckman-Coulter). The microbubbles were infused continuously into the jugular vein at 30 µl/min. Microbubbles were infused for 10 min before beginning data acquisition to allow the bubbles to reach steady state within the blood pool. Data acquisition was started 6 min before the start of saline, insulin, or insulin + L-NAME infusion. Images were acquired continuously at a pulsing interval of 20 s. Data were analyzed offline. Frames were aligned by cross-correlation, and several frames during a short pulsing interval (0.5 s) were averaged and subtracted from frames at a pulsing interval of 20 s. This background subtraction served to largely eliminate contributions from vessels with blood flow velocity faster than 5 mm/s (vessels >20 µm in diameter).

Western analysis. Samples of calf muscle, freeze-clamped at the end of the protocols, were powdered under liquid nitrogen. Approximately 20 mg of the tissue was added to cold 25 mmol/l Tris HCl buffer (26 mmol/l potassium fluoride and 5 mmol/l EDTA, pH 7.5). Tissue was then disrupted by sonication with the use of a microtip probe, 0.5 s on and 0.5 s off for 45 s total at a 3.0 power setting on the Fisher XL2020 sonicator. The homogenate was then centrifuged at 5,000 rpm for 5 min, the protein content of the supernatant was determined, and one aliquot containing 120 µg protein was heated to 100°C with an equal volume of SDS sample buffer and electrophoresed on an 8% polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes. After being blocked with 5% low-fat milk in Tris-buffered saline plus Tween 20, membranes were incubated with rabbit anti–phospho-Akt (Cell Signaling Technology, Beverly, MA) overnight at 4°C. This was followed by a 1-h incubation with anti-rabbit IgG coupled to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) at room temperature, and then the blot was developed using an enhanced chemiluminescence Western blotting kit (Amersham Biosciences). Autoradiographic film was scanned densitometrically (Molecular Dynamics, Piscataway, NJ) and quantitated using TotalLab software.

Statistical analysis. Two-way repeated-measures ANOVA was used to assess the effects of infusion protocols on microvascular volume. Post hoc testing was used as indicated in the results.

RESULTS

The plasma glucose concentrations, the glucose infusion rate during the clamp, and the femoral artery flow are shown in Fig. 1 for rats studied using each of the three protocols. Results for plasma glucose and glucose infusion rate were pooled for the two groups studied with each protocol because there were no differences between the two groups. Femoral flow was only measured in the rats not receiving microbubbles (see RESEARCH DESIGN AND METHODS). There were no significant time-dependent changes in either plasma glucose or femoral artery blood flow during the 30-min infusions (repeated-measures ANOVA). Femoral flow did appear slightly higher in the insulin + L-NAME, and this occurred together with an increment in systemic blood pressure of ~30 mmHg (compared with insulin alone), whereas vascular resistance of the hindlimb was not increased significantly over the other two study groups. The exogenous glucose infusion rate was similar in protocols 2 and 3 where insulin was given. At 30 min, the plasma insulin concentrations were as expected higher ($P < 0.01$) with insulin (530 ± 56 pmol/l) than with saline (~260 ± 37 pmol/l). There was no difference in plasma insulin between insulin and insulin + L-NAME infusions.

Figure 2 illustrates the time course for change in microvascular volume (presented as the fold-change over baseline) for the three study protocols. This was calculated by measuring video intensity every 20 s for 5 min before beginning insulin, saline, or insulin + L-NAME to obtain an average baseline video intensity. The average video intensity for subsequent 5-min blocks was divided by this baseline value. Insulin infusion provoked a time-dependent increase in the microvascular volume ($P < 0.001$, repeated-measures ANOVA) that was significant (55 ±
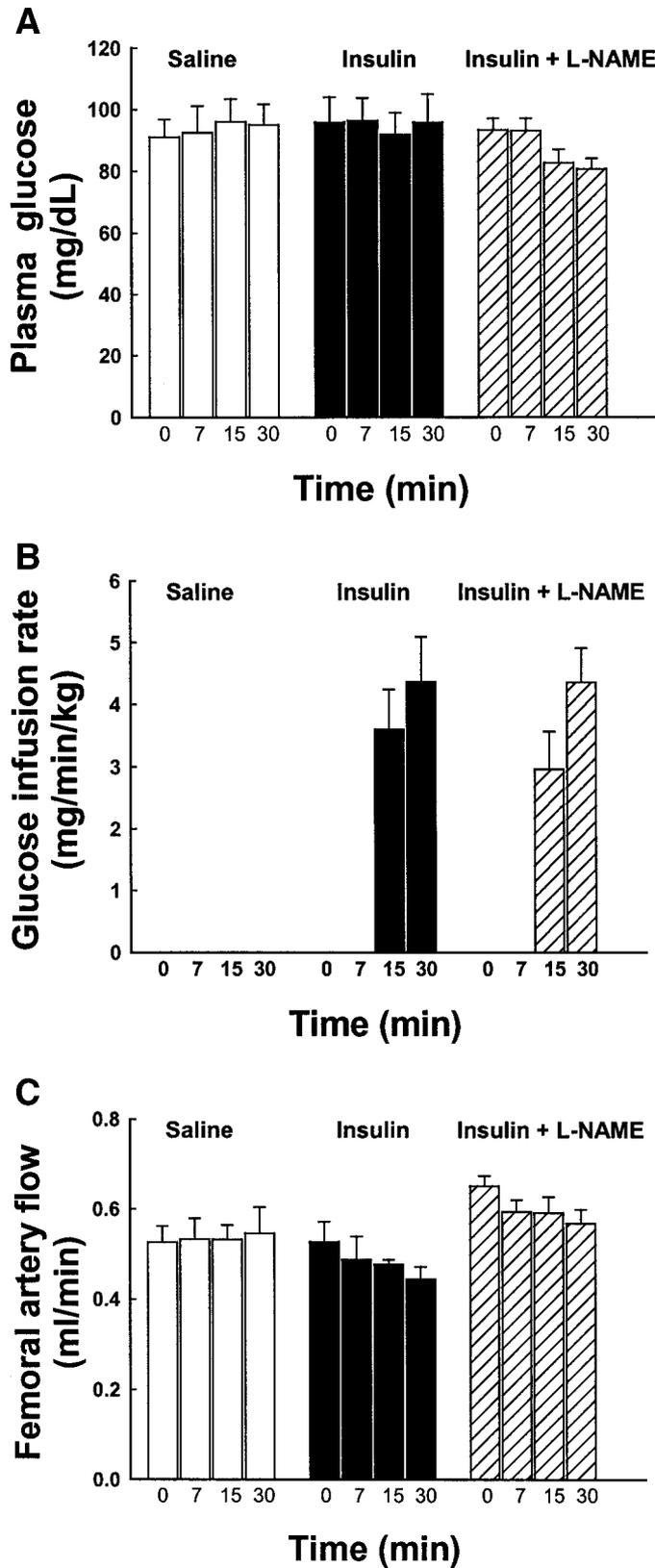
FIG. 1. The time course plasma glucose concentrations (*A*), exogenous glucose infusion (*B*), and femoral blood flow (*C*) in the three groups of animals. Data are means ± SE for six to eight rats in each group.
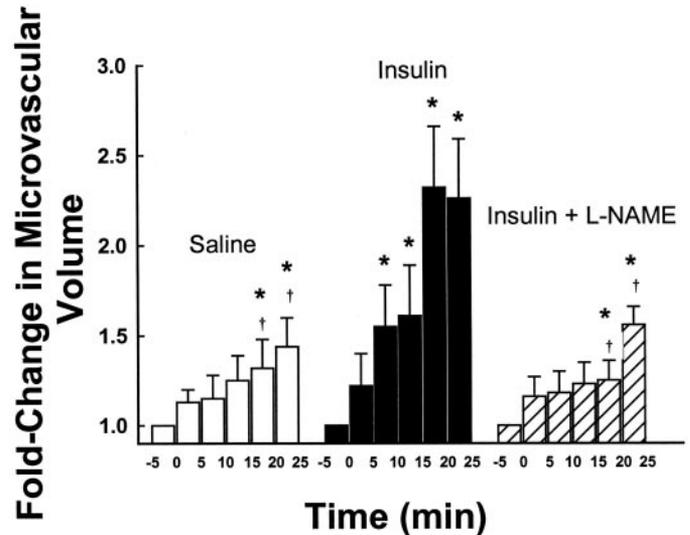

FIG. 2. Video intensity was measured every 20 s. The average for the interval between −5 and 0 min was considered the baseline and normalized to a value of 1, with each subsequent 5-min block given as the ratio to the baseline period. Video intensity curves for saline (□), insulin (3 mU · kg^{-1} · min^{-1} [■]), or insulin + L-NAME (50 µg · kg^{-1} · min^{-1} [▨]). Data are means of five to six animals in each group. *Significance from baseline (− 5 to 0 min) and saline (*P* < 0.05). †Significance from insulin (*P* < 0.05).

repeated-measures ANOVA). Microvascular volume also rose slowly in rats given L-NAME in addition to insulin (*P* < 0.01, repeated-measures ANOVA), and the pattern was not different than that seen with saline, possibly reflecting a response to the volume of fluid infused with the microbubbles. The increments in video intensity were significantly greater in the rats given insulin when compared with either of the other two groups (Fig. 2). By the end of the insulin infusion (25 min), microvascular volume in the insulin group had risen by 127 ± 33%.

We also measured the time course for the insulin-induced changes of arterial glucose concentration minus venous glucose concentration ($A_G − V_G$) across the rat hindleg. Because total limb blood flow did not change during the infusion period, $A_G − V_G$ directly indexes glucose disposal. Measurements were made at baseline (time = 0 min) and 7, 15, and 30 min. We observed that despite the increase in insulin-mediated microvascular blood volume between 5 and 10 min, the corresponding leg $A_G − V_G$ glucose disappearance did not significantly differ among saline, insulin, or insulin + L-NAME at baseline, 7 min, or even 15 min (Fig. 3). In addition, it was not necessary to infuse exogenous glucose to maintain arterial glucose constant until after 7 min into the insulin infusion (Fig. 1). By 30 min, $A_G − V_G$ during insulin infusion was 14 ± 3 mg/dl, which was significantly (two-way ANOVA) higher (*P* < 0.05) than baseline, 30-min saline (4 ± 1 mg/dl), or 30-min insulin + L-NAME (7 ± 1 mg/dl). These findings suggest that microvascular recruitment preceded increases in glucose disposal during insulin infusion. There was no significant difference in the glucose infusion rate required to maintain euglycemia between the insulin alone or insulin + L-NAME groups at either 15 or 30 min.

Before insulin can increase muscle glucose uptake, it must first activate insulin-signaling pathways in muscle (25). To address whether insulin-mediated capillary recruitment preceded or followed the activation of muscle

22%, *P* < 0.05) by 5–10 min after beginning the infusion. There were lesser and slower time-dependent increases in microvascular volume in the saline-infused rats (*P* < 0.01,
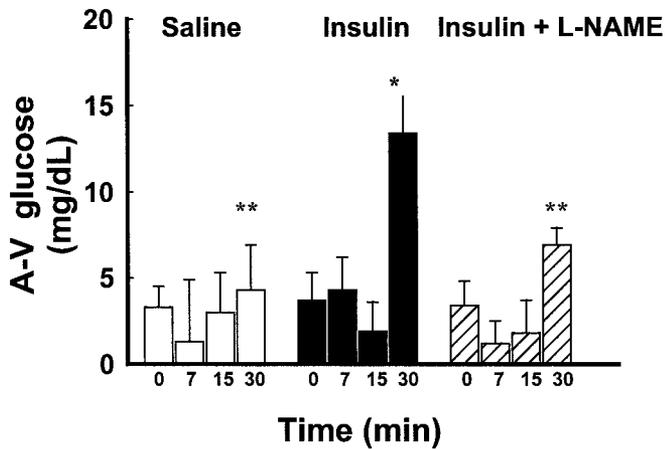
FIG. 3. Hindleg $A_G - V_G$ glucose difference (A-V glucose) was determined at baseline (time = 0 min) and 7, 15, and 30 min of saline (○) or insulin infusion (●) or insulin + L-NAME (50 µg · kg^{-1} · min^{-1}). Data are means ± SE ($n = 6–8$). *Significance from baseline ($P < 0.05$). **Significance from insulin ($P < 0.05$).

insulin signaling, we measured Akt phosphorylation in calf muscle freeze-clamped at 7 or 30 min of saline, insulin, or insulin + L-NAME infusion. Western analysis revealed (Fig. 4) that at 7 min, Akt phosphorylation was not significantly different between any of the groups. However, by 30 min, insulin, compared with saline, significantly ($P < 0.05$) enhanced Akt phosphorylation. Akt phosphorylation in insulin + L-NAME at 30 min was intermediate and not statistically different than insulin or saline alone. The nitrocellulose membranes were stripped and blotted for total Akt, and there were no differences among the three groups (data not shown). As mentioned previously, Akt phosphorylation in endothelial cells precedes activation of eNOS (12,26). However, endothelial tissue contributes negligibly to the mass of tissue analyzed here, and the
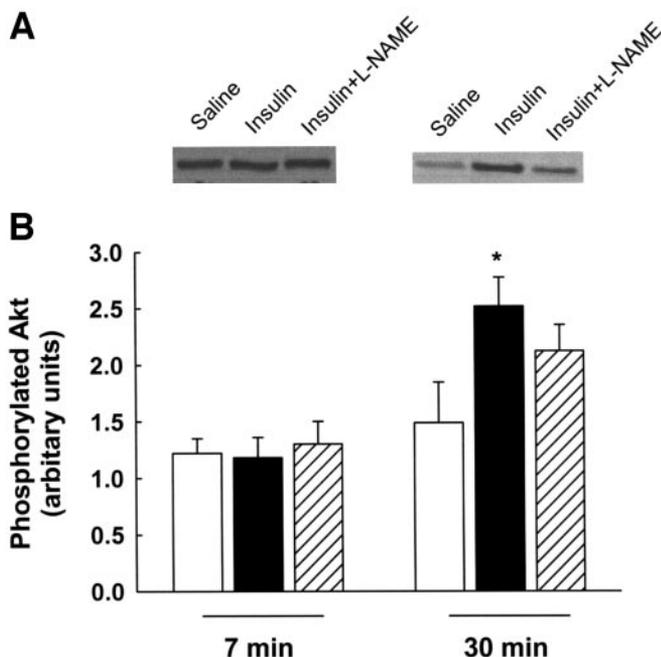

FIG. 4. Western analysis of freeze-clamped calf muscle Akt phosphorylation. A: Typical phospho-Akt bands determined by Western analysis. B: Average values from five to nine experiments. Data are means ± SE. *Significance from 30-min saline infusion ($P < 0.05$).

Akt measured in these whole muscle homogenates overwhelmingly reflects that of the myocyte.

DISCUSSION

The findings reported here demonstrate that, within minutes, insulin significantly increases the microvascular volume perfused in resting skeletal muscle. This is a distinctly different time course from that seen for the increase in total hindlimb blood flow in the rat (17) or for increases in leg or forearm blood flow in response to physiological hyperinsulinemia in humans (2). In both cases, these increases in total flow require 1 to several hours. The vessels involved in these responses are distinct. Capillary blood volume increases when precapillary arterioles dilate, allowing flow to previously unperfused or underperfused vessels. Total flow increases when larger resistance vessels relax. It has been previously demonstrated that these two classes of arterial vessels can be regulated independently in response to electrical stimulation (27). Low-frequency (four cycles per minute) electrical stimulation of the obturator nerve relaxes precapillary arterioles in canine skeletal muscle and recruits previously unperfused capillary beds in the absence of any changes in total blood flow (27). Higher-frequency stimulation both recruits microvasculature and increases total blood flow. With electrical stimulation, both responses occur promptly after stimulation. Likewise, we have observed that low-intensity forearm exercise increases microvascular volume measured by CEU without changing total forearm blood flow, whereas higher-intensity exercise increases both forearm flow and microvascular volume (28).

Considerable other data indicate that relaxation of precapillary arterioles and resistance vessels can be regulated independently. Previously, we reported that in human skeletal muscle, insulin infused locally into the brachial artery increased forearm microvascular volume without affecting total forearm flow (29). We have also recently measured the dose-response characteristics for insulin's action on total blood flow and on microvascular volume using both the CEU method used here (30) and measurements of 1-methylxanthine removal by hindlimb skeletal muscle (31). In those studies, regardless of the method used, microvascular recruitment was more sensitive than total blood flow to 2 h of steady-state hyperinsulinemia. Conversely, in the rat hindlimb in vivo, we observed that low doses of epinephrine increase total flow without affecting the capillary volume perfused (measured using either 1-methylxanthine [15] or laser Doppler flowmetry [14]).

In the current study, we show that the microvascular action of insulin, like that involving resistance vessels, can be blocked by inhibition of NOS. We had previously found that L-NAME blocked the increases in both total and hindlimb flow provoked by 2 h of marked hyperinsulinemia (10 mU · min^{-1} · kg^{-1}) in the rat hindlimb (18). However, in that study, both total flow and microvascular volume were increased by insulin; as a result, we could not discount the possibility that the decline in capillary recruitment caused by L-NAME was in some manner secondary to an effect to decrease total flow. Clearly, findings in the current study using lower insulin doses and shorter

observation times when total flow is unchanged supports a direct effect of NOS inhibition on microvascular recruitment.

It has not been addressed previously whether the vasodilatory effects of insulin on either resistance vessels or precapillary arterioles was a direct effect of insulin on the vascular cells or arose indirectly, mediated by either a metabolic action of insulin within the muscle, or perhaps neural action of insulin. Insulin receptors are present on endothelial cells (32), and insulin can activate NOS via a specific phosphorylation mediated by Akt (10). The fact that NOS inhibition was able to block both vascular actions of insulin is consistent with a direct endothelial effect of insulin but does not eliminate the possibility that an indirect action is contributing. This issue is particularly pertinent to the effect of insulin on resistance vessels simply because of the time that appears to be required to relax these vessels. Insulin can activate its receptor and downstream signaling to Akt and NOS within seconds in isolated endothelial cells (11,12) but requires 30 min to several hours to dilate resistance vessels (2,33). In contrast, the rapid time course for insulin's action to increase microvascular perfusion argues for a direct effect on the vasculature.

It is of interest to compare the time course of insulin's microvascular action reported here to its time course for its appearance in muscle interstitium, activation of insulin receptor kinase activity, and stimulation of glucose disposal. This has been carefully studied by Miles et al. (34), who estimated the half-times for insulin transfer from the vasculature to the muscle interstitium (~28 min), for activation of the receptor tyrosine kinase in muscle (~32 min), and for increases in muscle glucose disposal (~20 min). From Fig. 2, it is clear that the time course for insulin's recruitment of microvasculature in the rat temporally precedes these other effects and is consistent with a direct microvascular action of insulin. These findings do not eliminate the possibility of a neural-mediated insulin effect. However, the observation that locally infused insulin recruits microvasculature in human forearm muscle is somewhat against a central neural action.

A potential limitation of the current work is the use of anesthetized animals. Because any movement distorts the ultrasound image, it is not possible to conduct these studies in conscious animals. However, whereas the absolute estimates of muscle microvascular perfusion may be altered from what would be seen in the conscious animal, this should not impair our ability to compare the three groups of animals in this study.

The fact that inhibition of NOS blunted glucose uptake by muscle supports a relationship between insulin's microvascular and metabolic actions. Bonadonna et al. (35) previously pointed out (based on the analysis by Renkin [36]) that in as much as the extraction ratio for glucose across skeletal muscle is relatively low under basal conditions and during modest hyperinsulinemia, changes in total flow would be expected to minimally affect glucose disposal. Conversely, when insulin concentrations are high and muscle glucose uptake strongly stimulated (a high extraction ratio for glucose across the muscle), then increments in total blood flow would significantly affect muscle glucose disposal (2,4).

We have observed that several factors that antagonize insulin-mediated glucose disposal (e.g., infusion of free fatty acids [37], tumor necrosis factor-α [38], or α-methyl serotonin [16]) each blunt the effect of insulin to recruit muscle microvasculature. Likewise, we have observed that insulin's action to recruit microvasculature is impaired in the insulin-resistant Zucker rat (39). Thus, understanding the relationship between insulin sensitivity and its action to recruit microvasculature may be important to the overall understanding of insulin resistance.

The relation of insulin's vascular to its metabolic actions has also been explored using genetically altered mice. The eNOS knockout mouse is insulin resistant (8,9), as is the nNOS animal. A vascular endothelial cell–specific insulin receptor knockout mouse has been reported to have a normal phenotype with regard to vascular development but to express lower amounts of both eNOS and endothelin 1 (13). Significant insulin resistance in these latter animals was seen when they were on a low-salt diet but not a regular diet. Inasmuch as the specific changes in insulin sensitivity seen in these gene deletion studies may be affected by developmental changes, it is difficult to interpret these findings vis-à-vis normal physiological regulation. At present, the status of insulin-mediated capillary recruitment, or indeed vasodilation, generally in the muscle of these mice is unknown.

In conclusion, physiological hyperinsulinemia rapidly recruits microvascular elements in rat skeletal muscle in vivo. This vascular action of insulin precedes insulin's effects to increase muscle glucose uptake or activate downstream kinases involved in mediating skeletal muscle insulin action. Inhibition of NOS specifically blocks this action of insulin and diminishes muscle glucose uptake, reaffirming that this vascular action of insulin is partly responsible for insulin's overall metabolic action in muscle.

REFERENCES

1. Baron A: Hemodynamic actions of insulin. *Am J Physiol* 267:E187–E202, 1994
2. Yki-Jarvinen H, Utriainen T: Insulin-induced vasodilatation: physiology or pharmacology? *Diabetologia* 41:369–379, 1998
3. Taddei S, Virdis A, Mattei P, Natali A, Ferrannini E, Salvetti A: Effect of insulin on acetylcholine-induced vasodilation in normotensive subjects and patients with essential hypertension. *Circulation* 92:2911–2918, 1995
4. Steinberg HO, Brechtel G, Johnson A, Fineberg F, Baron AD: Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent: a novel action of insulin to increase nitric oxide release. *J Clin Invest* 94:1172–1179, 1994
5. Kobzik L, Reid M, Bredt D, Stamler J: Nitric oxide in skeletal muscle. *Nature* 372:546–548, 1994
6. Silvagno F, Xia H, Bredt D: Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J Biol Chem* 271:11204–11208, 1996
7. Segal SS, Brett SE, Sessa WC: Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters. *Am J Physiol* 277:H1167–H1177, 1999
8. Shankar RR, Wu Y, Shen HQ, Zhu JS, Baron AD: Mice with gene disruption of both endothelial and neuronal nitric oxide synthase exhibit insulin resistance. *Diabetes* 49:684–687, 2000
9. Duplain H, Burcelin R, Sartori C, Cook S, Egli M, Lepori M, Vollenweider P, Pedrazzini T, Nicod P, Thorens B, Scherrer U: Insulin resistance,

hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. *Circulation* 104:342–345, 2001

10. Zeng G, Quon MJ: Insulin-stimulated production of nitric oxide is inhibited by wortmannin: direct measurement in vascular endothelial cells. *J Clin Invest* 98:894–898, 1996

11. Zeng G, Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowski H, Quon MJ: Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation* 101:1539–1545, 2000

12. Montagnani M, Chen H, Barr VA, Quon MJ: Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179). *J Biol Chem* 276:30392–30398, 2001

13. Vicent D, Ilany J, Kondo T, Naruse K, Fisher SJ, Kisanuki YY, Bursell S, Yanagisawa M, King GL, Kahn CR: The role of endothelial insulin signaling in the regulation of vascular tone and insulin resistance. *J Clin Invest* 111:1373–1380, 2003

14. Clark ADH, Barrett EJ, Rattigan S, Wallis MG, Clark MG: Insulin stimulates laser Doppler signal by rat muscle in vivo consistent with nutritive flow recruitment. *Clin Sci* 100:283–290, 2001

15. Rattigan S, Clark MG, Barrett EJ: Hemodynamic actions of insulin in rat skeletal muscle: evidence for capillary recruitment. *Diabetes* 46:1381–1388, 1997

16. Rattigan S, Clark MG, Barrett EJ: Acute insulin resistance in rat skeletal muscle in vivo induced by vasoconstriction. *Diabetes* 48:564–569, 1999

17. Vincent MA, Dawson D, Clark AD, Lindner JR, Rattigan S, Clark MG, Barrett EJ: Skeletal muscle microvascular recruitment by physiological hyperinsulinemia precedes increases in total blood flow. *Diabetes* 51:42–48, 2002

18. Vincent MA, Barrett EJ, Lindner JR, Clark MG, Rattigan S: Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am J Physiol Endocrinol Metab* 285:E123–E129, 2003

19. Lindner JR, Song J, Jayaweera AR, Sklenar J, Kaul S: Microvascular rheology of Definity microbubbles after intra-arterial and intravenous administration. *J Am Soc Echocardiogr* 15:396–403, 2002

20. Wei K, Skyba DM, Firschke C, Jayaweera AR, Lindner JR, Kaul S: Interactions between microbubbles and ultrasound: in vitro and in vivo observations. *J Am Coll Cardiol* 29:1081–1088, 1997

21. Skyba DM, Price RJ, Linka AZ, Skalak TC, Kaul S: Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. *Circulation* 98:290–293, 1998

22. Lindner JR, Skyba DM, Goodman NC, Jayaweera AR, Kaul S: Changes in myocardial blood volume with graded coronary stenosis. *Am J Physiol* 272:H567–H575, 1997

23. Dawson D, Vincent MA, Barrett EJ, Kaul S, Clark A, Leong-Poi H, Lindner JR: Vascular recruitment in skeletal muscle during exercise and hyperinsulinemia assessed by contrast ultrasound. *Am J Physiol Endocrinol Metab* 282:E714–E720, 2002

24. Klibanov AL, Rasche PT, Hughes MS, Wojdyla JK, Galen KP, Wible JH Jr, Brandenburger GH: Detection of individual microbubbles of an ultrasound contrast agent: fundamental and pulse inversion imaging. *Acad Radiol* 9 (Suppl. 2):S279–S281, 2002

25. Patti ME, Kahn CR: The insulin receptor: a critical link in glucose homeostasis and insulin action. *J Basic Clin Physiol Pharmacol* 9:89–109, 1998

26. Luo Z, Fujio Y, Kureishi Y, Rudic RD, Daumerie G, Fulton D, Sessa WC, Walsh K: Acute modulation of endothelial Akt/PKB activity alters nitric oxide-dependent vasomotor activity in vivo. *J Clin Invest* 106:493–499, 2000

27. Honig CR, Odoroff CL, Frierson JL: Active and passive capillary control in red muscle at rest and in exercise. *Am J Physiol* 243:H196–H206, 1982

28. Jahn L, Vincent MA, Lindner JR, Barrett EJ: Modest exercise enhances human skeletal muscle perfusion by capillary recruitment in the absence of changes in total blood flow (Abstract). *Diabetes* 51 (Suppl. 2):A59, 2002

29. Coggins MP, Lindner J, Rattigan S, Fasy E, Jahn L, Kaul S, Barrett EJ: Physiologic hyperinsulinemia enhances human skeletal muscle perfusion by capillary recruitment. *Diabetes* 50:2682–2690, 2001

30. Vincent MA, Clerk LH, Barrett E: Skeletal muscle microvascular recruitment is more insulin sensitive than femoral blood flow or glucose disposal (Abstract). *Diabetes* 52 (Suppl. 1):A48, 2003

31. Zhang L, Richards SM, Rattigan S, Barrett E, Clark MG: Capillary recruitment in muscle is a highly sensitive event in insulin action in vivo (Abstract). *Diabetes* 52 (Suppl. 1):A48, 2003

32. King GL, Johnson SM: Receptor-mediated transport of insulin across endothelial cells. *Science* 227:1583–1586, 1985

33. Baron AD, Brechtel-Hook G, Johnson A, Cronin J, Leaming R, Steinberg HO: Effect of perfusion rate on the time course of insulin-mediated skeletal muscle glucose uptake. *Am J Physiol* 271:E1067–E1072, 1996

34. Miles PD, Levisetti M, Reichart D, Khursheed M, Moossa AR, Olefsky JM: Kinetics of insulin action in vivo: identification of rate-limiting steps. *Diabetes* 44:947–953, 1995

35. Bonadonna RC, Saccomani MP, Delprato S, Bonora E, Defronzo RA, Cobelli C: Role of tissue-specific blood flow and tissue recruitment in insulin-mediated glucose uptake of human skeletal muscle. *Circulation* 98:234–241, 1998

36. Renkin EM: B.W. Zweifach Award lecture: regulation of the microcirculation. *Microvasc Res* 30:251–263, 1985

37. Clerk LH, Rattigan S, Clark MG: Lipid infusion impairs physiologic insulin-mediated capillary recruitment and muscle glucose uptake in vivo. *Diabetes* 51:1138–1145, 2002

38. Youd JM, Rattigan S, Clark MG: Acute impairment of insulin-mediated capillary recruitment and glucose uptake in rat skeletal muscle in vivo by TNF-alpha. *Diabetes* 49:1904–1909, 2000

39. Wallis MG, Wheatley CM, Rattigan S, Barrett EJ, Clark AD, Clark MG: Insulin-mediated hemodynamic changes are impaired in muscle of Zucker obese rats. *Diabetes* 51:3492–3498, 2002