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Exercise Increases Human Skeletal Muscle Insulin Sensitivity via Coordinated Increases in Microvascular Perfusion and Molecular Signaling

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Insulin resistance is a major health risk, and although exercise clearly improves skeletal muscle insulin sensitivity, the mechanisms are unclear. Here we show that initiation of a euglycemic-hyperinsulinemic clamp 4 h after single-legged exercise in humans increased microvascular perfusion (determined by contrast-enhanced ultrasound) by 65% in the exercised leg and 25% in the rested leg ($P < 0.05$) and that leg glucose uptake increased 50% more ($P < 0.05$) in the exercised leg than in the rested leg. Importantly, infusion of the nitric oxide synthase inhibitor L-N^G-monomethyl-L-arginine acetate (L-NMMA) into both femoral arteries reversed the insulin-stimulated increase in microvascular perfusion in both legs and abrogated the greater glucose uptake in the exercised compared with the rested leg. Skeletal muscle phosphorylation of TBC1D4 Ser^{318} and Ser^{704} and glycogen synthase activity were greater in the exercised leg before insulin and increased similarly in both legs during the clamp, and L-NMMA had no effect on these insulin-stimulated signaling pathways. Therefore, acute exercise increases insulin sensitivity of muscle by a coordinated increase in insulin-stimulated microvascular perfusion and molecular signaling at the level of TBC1D4 and glycogen synthase in muscle. This secures improved glucose delivery on the one hand and increased ability to take up and dispose of the delivered glucose on the other hand.

Exercise improves metabolic control both via increasing muscle glucose uptake during muscle contractions by insulin-independent mechanisms and by increasing skeletal muscle insulin sensitivity after physical activity (1–4). Skeletal muscle remains more sensitive to insulin for 24–48 h after exercise in both rodents (1,5) and humans (3,6,7). At 3 to 4 h after a 60-min bout of single-legged exercise in humans, leg glucose uptake (LGU) during a euglycemic-hyperinsulinemic clamp (insulin infusion) is markedly increased compared with the rested leg (3,7). Importantly, acute exercise also increases skeletal muscle insulin sensitivity in people with type 2 diabetes (8).

Determining the mechanisms responsible for the increased skeletal muscle insulin sensitivity during the recovery from exercise is clinically important for several reasons. First, a reduction in skeletal muscle insulin sensitivity is an early event in the development of not only prediabetes, metabolic syndrome, and type 2 diabetes but is also associated with other conditions such as cardiovascular disease and some cancers (9,10). A greater understanding of the mechanisms behind the acute exercise-induced increase in skeletal muscle insulin sensitivity will provide important information to develop new therapeutics. Second, increased postexercise insulin sensitivity plays a role in hypoglycemia after prolonged exercise in patients with type 1 diabetes. Understanding the mechanism of action of this process may be

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useful in diminishing this clinical problem. Third, the increase in insulin sensitivity after exercise is necessary for the accelerated rate of muscle glycogen resynthesis after exercise (11,12). Given that muscle glycogen is a critical substrate for short intense exercise and also longer endurance exercise (13), a fuller understanding of the mechanism(s) regulating muscle glycogen resynthesis may facilitate new strategies for the exercise recovery process and, as a result, exercise performance in athletes.

Although this insulin-sensitizing effect of acute contraction/exercise has been known for many years, the mechanisms involved remain unclear. This has remained a conundrum, partly because of the reductionist methods that have been used to try to understand a very complex integrated phenomenon. The greater skeletal muscle insulin sensitivity several hours after acute exercise could be a result of muscle cellular events and/or greater glucose delivery. Surprisingly, the effect of acute exercise on skeletal muscle insulin signaling is quite modest. Indeed, there is little evidence of greater proximal skeletal muscle insulin signaling after acute exercise with, for example, no greater increases in phosphorylation of Akt compared with noncontracted muscle (3,6,14,15). There are, however, indications of distal skeletal muscle insulin signaling enhancements after acute exercise, in particular, greater phosphorylation of Akt substrate of 160 KDa (AS160, also referred to as TBC1D4) (14–16) and greater activation of glycogen synthase (GS) (3,17). This implies that after exercise, the previously contracted muscle is primed for an increase in glucose uptake in response to insulin; however, unless there is an accompanying increase in glucose delivery, it might be expected that maximal glucose uptake cannot be obtained. Indeed, insulin at physiological concentrations is a vasodilator that has been shown to increase microvascular perfusion at rest without increasing total blood flow (18,19). Increases in microvascular perfusion are considered to be a local redirection of blood flow from nonnutritive to nutritive (capillaries) flow channels (microvascular perfusion) (20). No study has directly examined whether the insulin-sensitizing effect after exercise includes vascular effects that increase glucose delivery to the muscle.

The aim of this study was to determine whether the insulin-sensitizing effect of prior acute exercise is associated with increases in skeletal muscle microvascular perfusion. We hypothesized that increased insulin sensitivity of skeletal muscle microvascular perfusion after exercise is a hitherto unrecognized necessary component of increased insulin sensitivity of glucose uptake in muscle.

RESEARCH DESIGN AND METHODS

Participants and Study Approval

Thirteen healthy male volunteers (Table 1) were enrolled in this study, which was approved by the Copenhagen Ethics Committee (H-3-2013-089) and conformed to the code of ethics of the World Medical Association. Leg mass and lean leg mass was measured by DEXA scanning (DPX-IQ Lunar; Lunar Corporation, Madison, WI). VO_2 peak was determined by an incremental test performed on a bike ergometer (Monark, Vansbro, Sweden), and VO_2 was measured using a MasterScreen CPX online system (Becton, Dickinson and Co., Franklin Lakes, NJ). Participants were familiarized for 20 min to one-legged knee-extensor exercise on two occasions, and an incremental peak one-legged knee-extensor power output test was performed on a separate day a minimum of 1 week before the experimental day. Two subjects participated twice (once with biopsies, once with measurement of microvascular perfusion, see below). Some data for those two subjects (leg balance data and glucose infusion rate [GIR] during the clamp) were therefore measured twice, and the mean values from the two trials in each subject were used as results.

Table 1—Subject characteristics (N = 13)

Age, years	25 ± 1
Height, m	1.81 ± 0.01
Weight, kg	76.3 ± 1.5
Body fat, %	16.4 ± 1.4
BMI, kg/m^2	23.2 ± 0.2
Leg mass	
Exercised leg, kg	12.9 ± 0.3
Rested leg, kg	12.7 ± 0.3
Lean leg mass	
Exercised leg, kg	10.2 ± 0.3
Rested leg, kg	10.0 ± 0.3
VO_2 peak, mL · kg^{-1} · min^{-1}	49.9 ± 1.6
Peak knee-extensor work load, W	50.5 ± 1.9

Before the experimental day, subjects refrained from exercise training for 48 h. On the morning of the experimental day, subjects consumed a light breakfast containing 40 g of oatmeal and 150 mL of low-fat milk (total energy 837 kJ) at 5:00 A.M. Subjects arrived at the laboratory by public transportation at 6:45 A.M. and performed 60-min one-legged knee extensor exercise (at 80% of peak work load, with three 5-min intervals at 100% peak work load), as described earlier (21). Subjects then rested supine for 4 h to provide sufficient time for the leg blood flow in the exercised leg to return to preexercise levels (not different to the rested leg). During the 4 h, catheters were inserted into the femoral artery and vein of both legs below the inguinal ligament (Pediatric Jugular Catheterization set; Arrow International, Reading, PA) under local anesthesia (xylocaine 1%; AstraZeneca, Albertslund, Denmark) for subsequent measurement of LGU and for femoral artery infusion of L-N^G-monomethyl-L-arginine acetate (L-NMMA) (Clinalfa basic; Bachem AG, Bubendorf, Switzerland).

Arterial blood pressure was monitored continuously via one of the arterial catheters using a pressure transducer interfaced to an IntelliVue MP5 monitor (Phillips Healthcare, Andover, MA). Polyethylene catheters were then placed in antecubital veins for infusion of insulin and glucose and for microbubble infusion when relevant. A catheter was inserted in the dorsal hand vein for blood

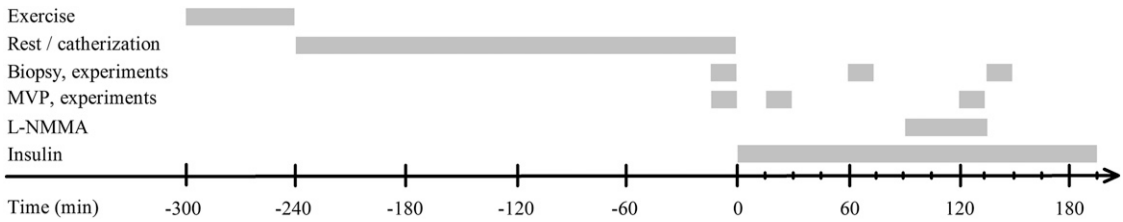

Figure 1—Schematic overview of the two protocols: protocol that included biopsies (Biopsy, experiments) and protocol that included microvascular perfusion (MVP, experiments). All other procedures occurred in both protocols.

sampling, and a heating pad was wrapped around the hand to arterialize the hand vein blood to oxygen saturation levels of between 93 and 96%. This was necessary because the femoral artery catheters were used for L-NMMA infusion, and therefore, blood samples could not be withdrawn simultaneously. A euglycemic-hyperinsulinemic clamp was initiated 4 h after the exercise was discontinued. Subjects were clamped at their individual ambient plasma glucose level obtained before initiation of the insulin infusion. The insulin infusion rate was 1.4 mU · kg^{-1} · min^{-1} for 195 min. The plasma glucose concentration was measured every 5–8 min, and a variable glucose infusion (20% Fresenius; Kabi, Uppsala, Sweden) into a forearm vein was adjusted to clamp euglycemia, as previously described (22). At 90 min into the clamp, L-NMMA (diluted into 50 mL saline) was infused at a constant rate (0.4 mg · kg^{-1} leg mass · min^{-1}) into both femoral arteries for 45 min (Fig. 1). The aim was to achieve decreases in leg blood flow with only very small systemic effects on blood pressure, similar to what we have previously observed during exercise (23) and others during insulin infusion (24) at this infusion rate. The insulin infusion was maintained for another 60 min after the L-NMMA infusion was discontinued (Fig. 1). During the clamp, blood samples were obtained every 15 min simultaneously from both femoral veins and the heated (arterialized) hand vein. Leg blood flow was measured before each blood sampling using a high frequency 9-3–MHz linear array transducer in power Doppler mode interfaced to an iU22 ultrasound machine (Phillips Ultrasound, Santa Ana, CA). In 9 of the 13 subjects, muscle biopsy specimens were obtained under local anesthesia from the vastus lateralis muscle of both legs before the clamp, after 60 min of insulin stimulation, and after 45 min of insulin plus L-NMMA infusion (Fig. 1) using a 5-mm Bergstrom needle with suction. In the other four subjects plus the two subjects that participated twice, microvascular perfusion in the vastus lateralis muscle of both legs was measured (Fig. 1). In one subject undergoing measurement of microvascular perfusion, femoral venous catheterization was not possible; hence, only microvascular data from this subject are added. Therefore, data presented are n = 12 for leg balance data, n = 9 for muscle specimen data, and n = 6 for the microvascular perfusion data.

Leg Blood Flow

Leg blood flow was measured using a high frequency 9-3–MHz linear array transducer in power Doppler mode. Diameter of the femoral artery was measured using two-dimensional imaging as the distance between inner arterial walls. Velocity was determined using pulse-wave Doppler, and the system calculated leg blood flow from the diameter and the velocity measurements.

Measurement of the Microvascular Perfusion

Microvascular perfusion in the vastus lateralis muscle was measured with a real-time contrast-enhanced ultrasound technique using an iU22 ultrasound system with an L9-3 transducer (Phillips Ultrasound) combined with infusion of Optison (GE Healthcare, Princeton, NJ) microbubbles, as described previously (25).

In short, a transducer was fixed to each thigh using an in-house manufactured strap-on device that kept it in the same place throughout the experiment and allowed for cross-sectional imaging of the vastus lateralis muscle. Optison microspheres were activated manually by shaking the vial for 3 min. Microbubbles (2 × 3 mL suspension) were diluted to 20 mL with sterile saline and infused intravenously at a rate of 1.5 mL · min^{-1} using a rotating syringe pump (VueJect, BR-INF100; Bracco, Geneva, Switzerland) to ensure a homogenous microbubble solution. To ensure systemic steady state, microbubbles were infused for 7 min before 30-s recordings in each leg in triplicate were performed to assess microvascular perfusion, as described previously (25). Real-time imaging was performed using a low mechanical index of 0.08, thereby allowing the microbubbles within the ultrasound beam to resonate without destruction. A high mechanical index of 1.20 was used at the beginning of each recording to destroy the microbubbles, thereby allowing recording of the replenishment of the microbubbles in the vasculature within the ultrasound beam. The acoustic intensity (AI) obtained during the first 0.5 s in the basal, insulin, and insulin + L-NMMA stimulated state were averaged and subtracted from the AI recorded during the remaining seconds, thereby eliminating background noise and the contribution from rapidly filling vessels (i.e., arteries, veins and large arterioles, or venules). Calculations for the microvascular perfusion were made in accordance with Wei et al. (26), where AI versus time curves were fitted to the exponential function: $y = A(1 - e^{-\beta[t-B_t]})$, where t is time (s), B_t is the time used for background subtraction, y is the acoustic intensity at any given t, A is the plateau AI, defined as an index of microvascular perfusion, and β is the flow rate constant (1/s) that determines the rate of rise of AI (26). Microvascular

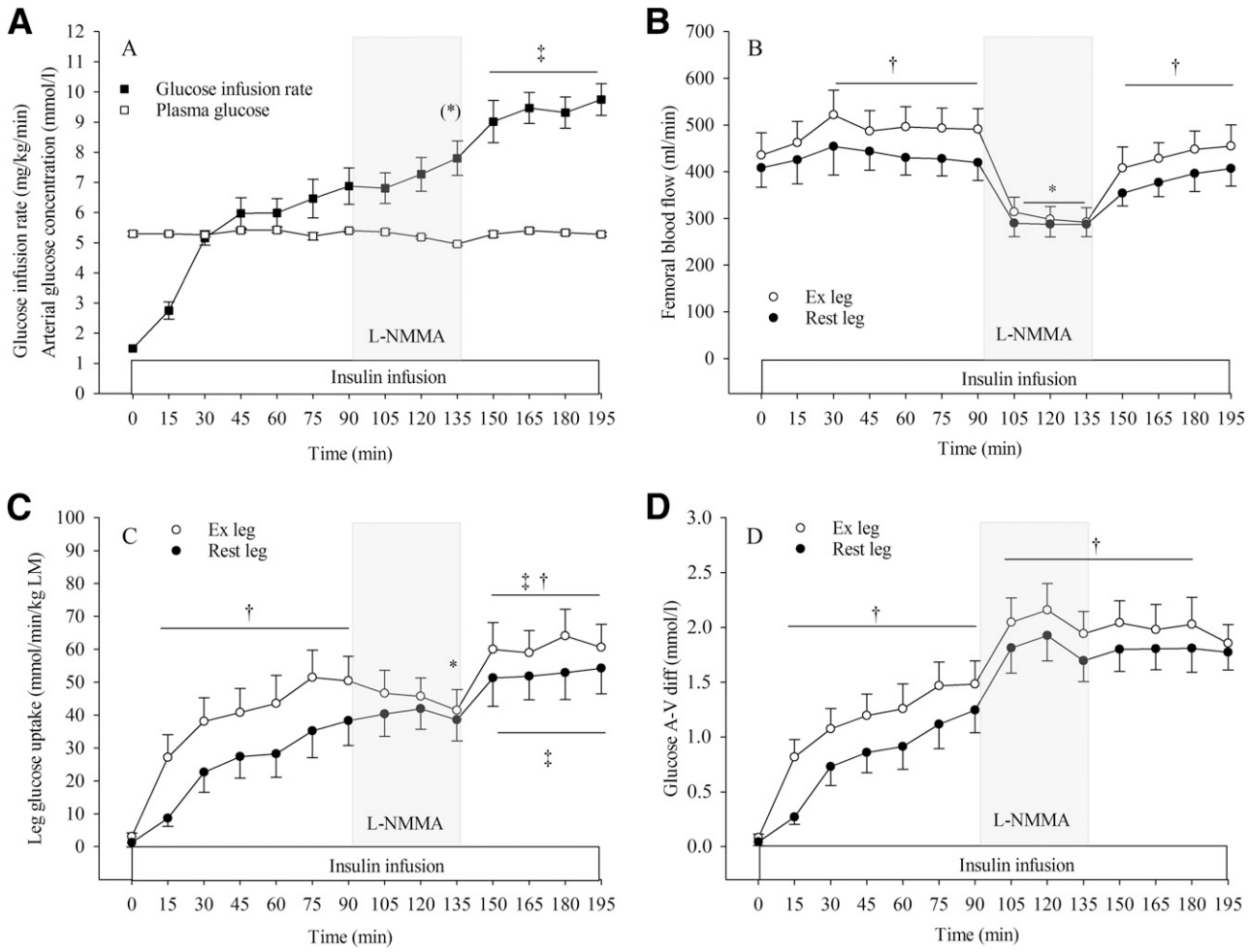

Figure 2—Euglycemic-hyperinsulinemic clamp, leg blood flow, leg a-v glucose concentration difference, and LGU. *A*: Arterialized plasma glucose concentration and GIR. *B*: Leg blood flow. *C*: LGU. *D*: The a-v glucose concentration difference (a-v diff). LM, leg mass. Data are presented as means ± SEM, $n = 12$. †$P < 0.05$ exercised (Ex) leg vs. rested (Rest) leg, *$P < 0.05$ vs. 90 min, ‡$P < 0.05$ vs. 135 min, (*) $P = 0.05$ vs. 90 min.

perfusion was measured before insulin, after 25 min of insulin infusion, and after 40 min of L-NMMA infusion (Fig. 1).

Blood Analysis and LGU

Plasma glucose concentrations during the experiments were measured on an ABL 800 FLEX (Radiometer Medical A/S, Copenhagen, Denmark). Plasma insulin was measured using an enzyme-linked immunosorbent assay (ALPCO, Salem, NH). LGU was calculated as the glucose concentration difference between the arterialized and the femoral venous blood multiplied by the leg blood flow.

SDS-PAGE and Western Blot Analyses

Vastus lateralis muscle (30 mg) was homogenized in ice-cold buffer, and lysates were prepared as previously described (27). All samples were heated in Laemmli buffer, and then SDS-PAGE and immunoblotting were performed for protein expression and protein phosphorylation. Antibodies used were anti-Akt2 (Cell Signaling Technology, Danvers, MA), anti-TBC1D4 (Upstate Biotechnology), AMPK-α2 (Santa Cruz Biotechnology), and anti–acetyl-CoA carboxylase (ACC)-β (Dako). The primary phosphorylated (p)-specific antibodies were anti–p-Akt Ser^{473} (Cell Signaling Technology), anti–p-Akt Thr^{308} (Cell Signaling Technology), anti–p-TBC1D4 Ser^{318} (Cell Signaling Technology), anti–p-TBC1D4 Ser^{704} (provided by L. Goodyear, Joslin Diabetes Center, Boston, MA), anti–p-TBC1D4 Thr^{642} (Cell Signaling Technology), anti–p-AMPK Thr^{172} (Cell Signaling Technology), and anti–p-ACC-β Ser^{221} (Cell Signaling Technology).

Muscle Glycogen and GS Activity

Muscle glycogen concentration was determined as glycosyl units after acid hydrolysis of freeze- dried and dissected muscle tissue by a fluorometric method (28). GS activity was measured in muscle homogenates by using a Unifilter 350 microtiter plate assay (Whatman; Frisenette, Ebeltoft, Denmark), essentially as described by Thomas et al. (29) modified for microtiter plate assay.

Statistical Analysis

Data are expressed as means ± SEM. Statistical evaluation involved two-way repeated-measures ANOVA (two

Table 2—Heart rate and blood pressure during the euglycemic-hyperinsulinemic clamp

Time (min)	Heart rate (bpm)	Systole (mmHg)	Diastole (mmHg)
0	58 ± 3	121 ± 3	63 ± 2
15	60 ± 3	121 ± 3	61 ± 2
30	62 ± 3	121 ± 3	61 ± 3
45	62 ± 3	119 ± 3	60 ± 2
60	62 ± 3	121 ± 3	62 ± 2
75	61 ± 2	121 ± 3	62 ± 2
90	61 ± 2	119 ± 3	62 ± 2
105	58 ± 3*†	126 ± 4*†	67 ± 2*†
120	58 ± 3*†	128 ± 3*†	70 ± 2*†
135	57 ± 2*†	129 ± 3*†	69 ± 2*†
150	59 ± 2	128 ± 3*	69 ± 2*
165	61 ± 3	127 ± 3*	70 ± 2*
180	62 ± 3	126 ± 4*	71 ± 2*
195	59 ± 3	124 ± 4*	69 ± 2*

Data are presented as means ± SEM, n = 12. †Indicates NOS inhibition. *$P < 0.05$ vs. 90.

factors: exercise and L-NMMA) after a Shapiro-Wilk normality test. When ANOVA revealed significant differences <0.05, specific differences were determined by a Tukey post hoc test.

RESULTS

Euglycemic-Hyperinsulinemic Clamp

Arterial plasma glucose was maintained at the baseline levels of 5.3 ± 0.1 mmol · L^{-1} (coefficient of variation = 4.7 ± 0.4%) throughout the experiment (Fig. 1 and Fig. 2*A*). L-NMMA was infused locally at a low dose into both femoral arteries to minimize systemic effects. However, there were small significant increases in systolic and diastolic blood pressure during the L-NMMA infusions, and even 60 min after cessation of the infusion, diastolic blood pressure remained slightly but significantly higher than before the L-NMMA infusion (Table 2). L-NMMA also decreased ($P < 0.05$) heart rate by ~4 bpm due to baroreceptor reflex (Table 2). The GIR averaged 6.9 ± 0.6 mg · min^{-1} · kg^{-1} after 90 min of insulin infusion (Fig. 2*A*). The GIR tended ($P = 0.05$) to increase during the 45 min of L-NMMA infusion (Fig. 2*A*). The GIR increased rapidly after the L-NMMA infusion was discontinued and reached ~9.5 mg · min^{-1} · kg^{-1} ($P < 0.05$) and remained essentially unchanged during the last 60 min of the experiment (Fig. 2*A*).

Leg Blood Flow

Femoral artery blood flow was similar 4 h after exercise in the previously exercised leg and the rested leg, demonstrating that the stimulatory effects of the prior exercise were no longer apparent (436 ± 48 mL · min^{-1} vs. 408 ± 41 mL · min^{-1}, respectively) (Fig. 2*B*). Insulin infusion for 90 min did not significantly increase leg blood flow in either leg, but leg blood flow was higher ($P < 0.05$) in the previously exercised leg compared with the rested leg from 30 min of insulin stimulation until commencement of the L-NMMA infusion (Fig. 2*B*). Infusion of L-NMMA into the femoral artery of both legs resulted in a rapid reduction in the blood flow to ~290 mL · min^{-1} ($P < 0.05$) in both legs. Leg blood flow then increased rapidly after the L-NMMA infusion was discontinued, reaching levels not significantly different from the pre–L-NMMA levels within 45 min in both legs (Fig. 2*B*). Leg blood flow was higher in the previously exercised leg compared with the rested leg ($P < 0.05$) after the L-NMMA infusion was discontinued (Fig. 2*B*).

LGU

LGU was similar in the previously exercised leg and the rested leg 4 h after exercise, indicating that the acute effect of exercise to raise LGU was no longer present (Fig. 2*C*). Insulin infusion increased LGU ($P < 0.05$) in both legs and more so (~50%) in the previously exercised leg ($P < 0.05$) (Fig. 2*C*). Infusion of L-NMMA into the femoral artery reduced LGU in the previously exercised leg from 50.4 ± 7.4 μmol · kg leg $mass^{-1}$ · min^{-1} at 90 min to 41.5 ± 6.3 μmol · kg leg $mass^{-1}$ · min^{-1} at 135 min ($P < 0.05$) and had no significant effect in the rested leg (Fig. 2*C*). In fact, L-NMMA in the previously exercised leg reduced LGU so that it was not significantly different from the rested leg (Fig. 2*C*). After the L-NMMA infusion was discontinued, LGU rapidly increased in both legs, and both exceeded the pre–L-NMMA levels within 15 min, and LGU in the previously exercised leg again became significantly higher ($P < 0.05$) than in the rested leg (Fig. 2*C*).

Arteriovenous Glucose Difference

The arteriovenous (a-v) difference was similar in the previously exercised leg and the rested leg 4 h after exercise (t 0 min of insulin infusion) (Fig. 2*D*). Insulin infusion increased the a-v difference ($P < 0.05$) in both legs, with the a-v difference increasing more in the previously exercised leg compared with the rested leg ($P < 0.05$) (Fig. 2*D*). Infusion of L-NMMA into the femoral artery increased the a-v difference ($P < 0.05$) in both legs (Fig. 2*D*), likely caused by the significant decrease in leg blood flow allowing for greater glucose extraction. Leg oxygen extraction increased from 23 ± 3% before to an average of 37 ± 3% ($P < 0.05$) during L-NMMA infusion, indicating an increase in capillary mean transit time. The glucose a-v difference remained elevated ($P < 0.05$) in the previously exercised leg compared with the rested leg during and after the L-NMMA infusion and until the 180-min time point (Fig. 2*D*). The a-v difference remained elevated after the L-NMMA infusion was discontinued compared with the pre–L-NMMA time point (90 min) in both legs (Fig. 2*D*).

Plasma Insulin

Insulin infusion of 1.4 mU · min^{-1} · kg^{-1} increased the plasma insulin concentration from 3.9 ± 0.7 to

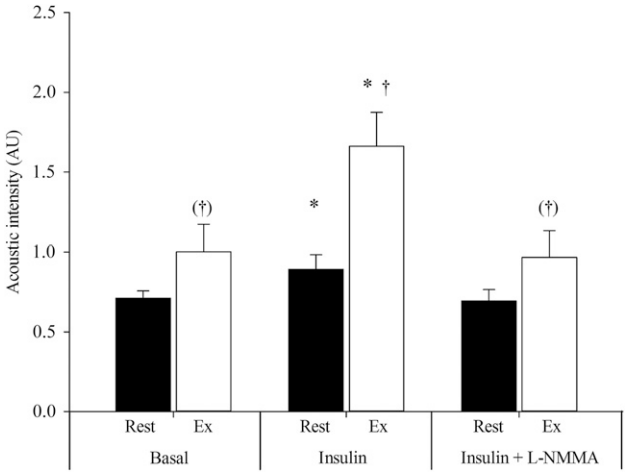

Figure 3—Microvascular perfusion at the basal level, during insulin infusion, and during insulin infusion with L-NMMA. Data are presented as means ± SEM, $n = 6$. †$P < 0.05$ exercised leg (Ex) vs. rested leg (Rest) with insulin, (†)$P = 0.056$ (basal) and $P = 0.087$ (insulin + L-NMMA). Partitioning the ANOVA revealed that the exercised leg was higher ($P < 0.05$) than the rested leg at the basal level. *$P < 0.05$ insulin infusion vs. basal and insulin infusion vs. insulin + L-NMMA. AU, arbitrary units.

103 ± 2.3 μIU · mL^{-1} within 30 min. Plasma insulin concentration rose by ~10% after 45 min of L-NMMA infusion ($P < 0.05$). This increase in plasma insulin was maintained for an additional 15 min after the L-NMMA infusion was discontinued.

Microvascular Perfusion

Microvascular perfusion was higher in the previously exercised leg than in the rested leg in all six subjects (Fig. 3). Compared with baseline, the increase in microvascular perfusion with insulin was more pronounced ($P < 0.05$) in the exercised leg compared with the rested leg (65% vs. 25%) (Fig. 3). The L-NMMA infusion reduced insulin-stimulated microvascular perfusion to levels almost identical to, and not different from, the basal level in both legs (Fig. 3).

Insulin Signaling in Skeletal Muscle

At 4 hours after exercise, basal levels of Akt Thr^{308} and Ser^{473} phosphorylation were similar in the two legs (Fig. 4*A* and *B*). Insulin infusion increased ($P < 0.05$) p-Akt Thr^{308} and p-Akt Ser^{473} to a similar extent in both legs (Fig. 4*A* and *B*). The combined insulin and L-NMMA infusion increased ($P < 0.05$) Akt Thr^{308} phosphorylation further in both legs ($P < 0.05$) (Fig. 4*A*), with no effect on Ser^{473} phosphorylation (Fig. 4*B*). Total Akt2 protein was similar between legs and time points (Fig. 4*C*).

There was a main effect ($P < 0.05$) for p-TBC1D4Ser^{704} and p-TBC1D4 Ser^{318} being higher in the previously exercised leg compared with the rested leg at all time points (Fig. 4*D* and *G*). Basal levels of p-TBC1D4 Thr^{642} and Ser^{588}were similar in the two legs (Fig. 4*E* and *F*). Insulin infusion increased p-TBC1D4 Ser^{704}, Thr^{642}, Ser^{318}, and Ser^{588} ($P < 0.05$) in both legs (Fig. 4*D*–*G*). The addition of L-NMMA did not affect insulin-stimulated p-TBC1D4 (Fig. 4*D*–*G*). Total TBC1D4 protein expression was similar in both legs at all time points (Fig. 4*H*).

There was a main effect of exercise on p-AMPKThr^{172} ($P < 0.05$) (Fig. 4*I*) and on p–ACC-β Ser^{221} ($P < 0.05$) (Fig. 4*K*), respectively. Total protein expression of AMPKα2 (Fig. 4*J*) and ACC-β (Fig. 4*L*) were similar in both legs at all time points.

Muscle Glycogen

Four hours after one-legged knee extensor exercise, muscle glycogen content was ~50% lower ($P < 0.05$) than in the rested leg (Fig. 5*A*). After 135 min of insulin and insulin + L-NMMA infusion, muscle glycogen content increased by ~30% ($P < 0.05$) compared with basal in the previously exercised leg only (Fig. 5*A*). Muscle glycogen content remained lower in the previously exercised leg compared with the rested leg throughout the experiment (Fig. 5*A*).

GS Activity

At 4 h postexercise, the GS I-form was ~150% higher ($P < 0.05$) in the previously exercised leg compared with the rested leg (Fig. 5*B*). Insulin infusion increased the GS I-form by ~60% ($P < 0.05$) in both legs, and the addition of L-NMMA had no effect (Fig. 5*B*). The GS fractional velocity followed a similar response to the GS I-form (Fig. 5*C*).

DISCUSSION

We have demonstrated that the ability of insulin to increase microvascular perfusion is augmented several hours after acute exercise in human skeletal muscle compared with nonexercise conditions. Importantly, this response was necessary to improve insulin sensitivity for glucose uptake in muscle after exercise. Our results suggest that acute exercise primes the previously active skeletal muscle for greater postexercise insulin-stimulated glucose uptake by two coordinated mechanisms: *1*) by increasing the response of the microvasculature to insulin, thereby enhancing microvascular perfusion and thus enabling an increased local glucose delivery to the muscle, and *2*) by preparing the muscle cell for an increased ability to take up and dispose of the delivered glucose at the level of TBC1D4 and GS.

Another novel finding was that despite similar blood flow in the two legs 4 h after exercise (noninsulin stimulated), microvascular perfusion was 40% higher in the exercised leg than in the rested leg. In addition, there was also greater TBC1D4 phosphorylation and muscle GS activity present within the exercised leg. Despite this, there was no greater LGU in the exercised leg, suggesting that a higher local glucose delivery and a priming of the glucose uptake machinery is insufficient to increase glucose uptake in the absence of increases in insulin concentration. These observations are in line with nonexercise models, such as glucagon-like peptide 1 administration, which increases microvascular perfusion but with no concomitant increased insulin concentration and skeletal muscle glucose uptake in humans and rats (30).

During insulin infusion, glucose uptake increased in both legs and to a greater extent in the exercised leg. For a

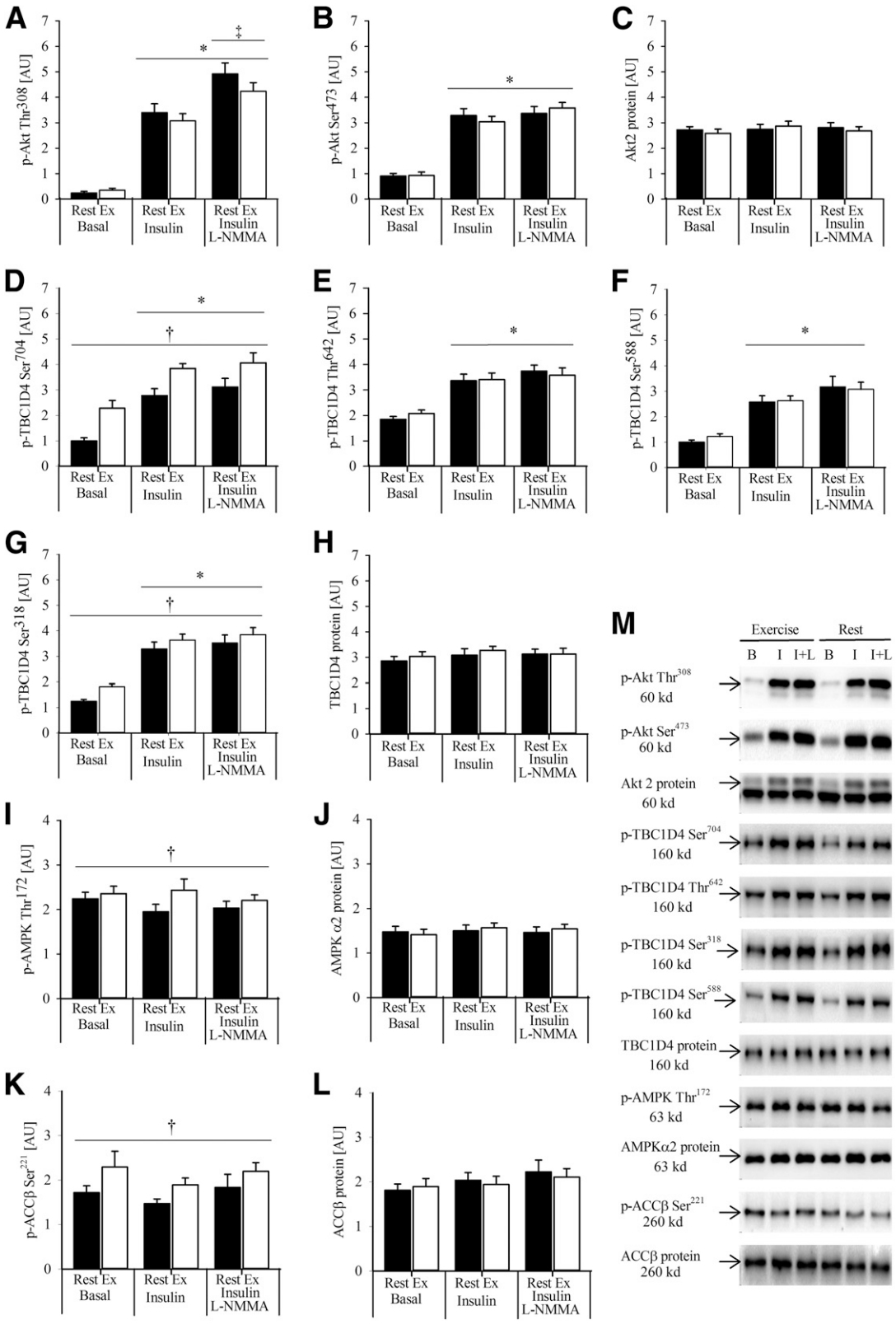

Figure 4—Insulin signaling and AMPK signaling in skeletal muscle before and during the euglycemic-hyperinsulinemic clamp: p-Akt Thr^{308} (*A*), p-Akt Ser^{473} (*B*), p-TBC1D4 Ser^{704} (*D*), p-TBC1D4 Ser^{642} (*E*), p-TBC1D4 Ser^{588} (*F*), p-TBC1D4 Ser^{318} (*G*), p-AMPK Thr^{172} (*I*), and p-ACC-β Ser^{221} (*K*). Protein expression of Akt2 (*C*), TBC1D4 (*H*), AMPK-α2 (*J*), and ACC-β (*L*) were similar between legs at all time points. *M*: Representative blots. Data are presented as means ± SEM, $n = 9$. †$P < 0.05$ exercised leg (Ex) vs. rested leg (Rest) (main effect), *$P < 0.05$ insulin infusion vs. basal, ‡$P < 0.05$ insulin + L-NMMA infusion higher than insulin infusion alone. AU, arbitrary units.

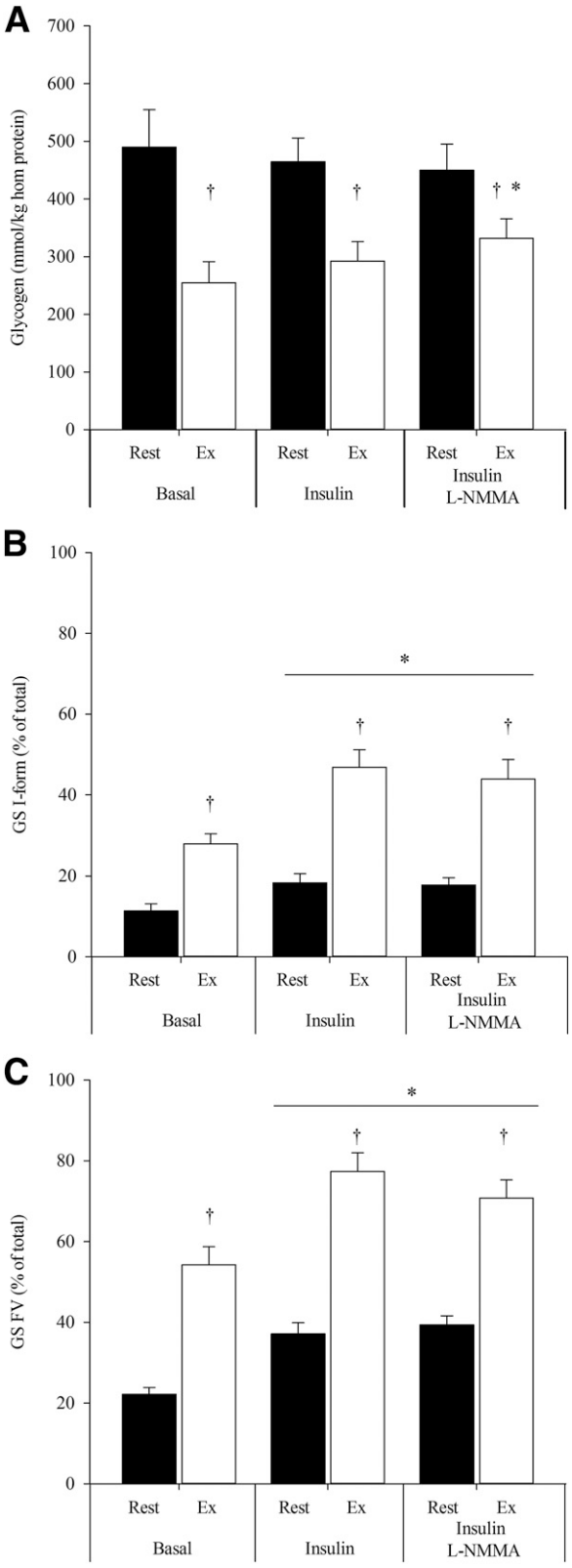

Figure 5—Muscle glycogen and GS. Muscle glycogen content (*A*), GS I-form (*B*), and GS fractional velocity (FV) (*C*). Data are presented as means ± SEM, $n = 9$. †$P < 0.05$ exercised leg (Ex) vs. rested leg (Rest) (main effect), *$P < 0.05$ insulin infusion vs. basal. Hom, homogenate.

muscle to take up more glucose, there must be both signals within the muscle related to GLUT-4 translocation to the cell membrane and increased glucose disposal and an increase in glucose delivery. Most studies of signaling within the muscle cells, including the present, find little effect of prior exercise or contraction on proximal insulin signaling such as Akt phosphorylation and insulin receptor substrate 1 activity (3,31–33). However, there is evidence that there is greater distal skeletal muscle insulin signaling at the level of TBC1D4 3–4 h after contraction/exercise in rats and humans (14,15,34). Indeed, we also found greater p-TBC1D4 Ser^{318} and p-TBC1D4 Ser^{704} 4 h after exercise in the previously exercised leg. This suggests that exercise primed the skeletal muscle for greater insulin-stimulated glucose uptake likely at the level of GLUT-4 translocation, resulting in an amplified response once insulin was increased.

The relationship between leg blood flow and LGU without prior exercise was studied by Baron et al. (35) in the 1990s, demonstrating that insulin infusion for 240 min increased leg blood flow by twofold. However, increases in leg blood flow are not always observed during 60–180 min of insulin infusion (25,35–37). In the current study, insulin increased the microvascular perfusion in both legs, and the increase was substantially greater in the previously exercised leg despite minor differences in total leg blood flow. Previous studies have also shown that insulin at physiological concentrations may increase microvascular perfusion without increasing total blood flow (18,19,25). This is thought to occur via a local redirection of blood flow from nonnutritive to nutritive flow channels (20).

The mechanism(s) responsible for the greater microvascular perfusion after exercise may be an increase in endothelial function at the level of endothelial nitric oxide synthase (NOS). Remarkably, NOS inhibition during the insulin infusion attenuated LGU in the exercised leg such that it was not different from the rested leg. This is a novel observation that has not previously been examined in any species. The reduction in insulin-stimulated glucose uptake in the previously exercised leg with NOS inhibition was likely caused by the decrease in glucose delivery because microvascular perfusion was lowered to preinsulin levels and leg blood flow was reduced by 40%, which was below the preinsulin levels. Similar to the current study in which the exercised leg was more insulin sensitive compared with the rested leg, Baron et al. (35) observed the action of both insulin and L-NMMA infusion on blood flow and LGU occurred within the most insulin-sensitive subjects. Notably, this reduction in LGU uptake in the exercised leg occurred without any effect of NOS inhibition on skeletal muscle insulin signaling.

During NOS inhibition, the large decrease in leg blood flow and microvascular perfusion in both legs was accompanied by an increase in leg a-v glucose difference in both legs, similar to that observed by Baron et al. (35). Given that the skeletal muscle insulin signaling within the

cell was maintained, this indicates that there must have been an increase in mean transit time of blood through the open capillaries allowing for greater glucose extraction, as also indicated by the leg oxygen extraction, which increased during L-NMMA infusion. The increase in leg a-v glucose difference was sufficient to compensate for the lower glucose delivery because LGU in the rested leg was maintained but was insufficient to compensate for the reduction in glucose delivery in the exercised leg. This indicates that glucose delivery had become the limiting factor in the exercised leg. This notion is supported by the fact that there was no effect of NOS inhibition on skeletal muscle insulin signaling.

In conclusion, we have shown that increases in glucose delivery are required for normal postexercise increases in skeletal muscle insulin sensitivity of glucose uptake in normal healthy men. In addition, the previously exercised muscle had higher distal insulin signaling at the level of TBC1D4 and GS activity, and it appears that this combination of priming of the muscle signaling and microvascular perfusion allows for increased skeletal muscle insulin sensitivity after exercise. These novel results are important as they provide an integrated understanding of how acute exercise increases skeletal muscle insulin sensitivity in humans.

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References

1. Richter EA, Garetto LP, Goodman MN, Ruderman NB. Muscle glucose metabolism following exercise in the rat: increased sensitivity to insulin. J Clin Invest 1982;69:785–793

2. Richter EA, Derave W, Wojtaszewski JF. Glucose, exercise and insulin: emerging concepts. J Physiol 2001;535:313–322

3. Wojtaszewski JF, Hansen BF, Gade J, et al. Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. Diabetes 2000;49:325–331

4. Ploug T, Galbo H, Richter EA. Increased muscle glucose uptake during contractions: no need for insulin. Am J Physiol 1984;247:E726–E731

5. Funai K, Schweitzer GG, Sharma N, Kanzaki M, Cartee GD. Increased AS160 phosphorylation, but not TBC1D1 phosphorylation, with increased postexercise insulin sensitivity in rat skeletal muscle. Am J Physiol Endocrinol Metab 2009;297:E242–E251

6. Wojtaszewski JF, Richter EA. Effects of acute exercise and training on insulin action and sensitivity: focus on molecular mechanisms in muscle. Essays Biochem 2006;42:31–46

7. Richter EA, Mikines KJ, Galbo H, Kiens B. Effect of exercise on insulin action in human skeletal muscle. J Appl Physiol (1985) 1989;66:876-885.

8. Devlin JT, Hirshman M, Horton ED, Horton ES. Enhanced peripheral and splanchnic insulin sensitivity in NIDDM men after single bout of exercise. Diabetes 1987;36:434–439

9. Orgel E, Mittelman SD. The links between insulin resistance, diabetes, and cancer. Curr Diab Rep 2013;13:213–222

10. Laakso M, Kuusisto J. Insulin resistance and hyperglycaemia in cardiovascular disease development. Nat Rev Endocrinol 2014;10:293–302

11. McCoy M, Proietto J, Hargreaves M. Skeletal muscle GLUT-4 and postexercise muscle glycogen storage in humans. J Appl Physiol (1985) 1996;80:411–415.

12. Jensen TE, Richter EA. Regulation of glucose and glycogen metabolism during and after exercise. J Physiol 2012;590:1069–1076

13. Romijn JA, Coyle EF, Sidossis LS, et al. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. Am J Physiol 1993;265:E380–E391

14. Treebak JT, Pehmøller C, Kristensen JM, et al. Acute exercise and physiological insulin induce distinct phosphorylation signatures on TBC1D1 and TBC1D4 proteins in human skeletal muscle. J Physiol 2014;592:351–375

15. Kjøbsted R, Munk-Hansen N, Birk JB, et al. Enhanced muscle insulin sensitivity after contraction/exercise is mediated by AMPK. Diabetes 2016;66:598–612

16. Pehmøller C, Brandt N, Birk JB, et al. Exercise alleviates lipid-induced insulin resistance in human skeletal muscle-signaling interaction at the level of TBC1 domain family member 4. Diabetes 2012;61:2743–2752

17. Pedersen AJ, Hingst JR, Friedrichsen M, Kristensen JM, Højlund K, Wojtaszewski JF. Dysregulation of muscle glycogen synthase in recovery from exercise in type 2 diabetes. Diabetologia 2015;58:1569–1578

18. Vincent MA, Clerk LH, Lindner JR, et al. Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. Diabetes 2004;53:1418–1423

19. Vincent MA, Dawson D, Clark AD, et al. Skeletal muscle microvascular recruitment by physiological hyperinsulinemia precedes increases in total blood flow. Diabetes 2002;51:42–48

20. Baron AD, Clark MG. Role of blood flow in the regulation of muscle glucose uptake. Annu Rev Nutr 1997;17:487–499

21. Kiens B, Essen-Gustavsson B, Christensen NJ, Saltin B. Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. J Physiol 1993;469:459–478

22. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 1979;237:E214–E223

23. Kingwell BA, Formosa M, Muhlmann M, Bradley SJ, McConell GK. Nitric oxide synthase inhibition reduces glucose uptake during exercise in individuals with type 2 diabetes more than in control subjects. Diabetes 2002;51:2572–2580

24. Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. J Clin Invest 1994;94:1172–1179

25. Sjøberg KA, Rattigan S, Hiscock N, Richter EA, Kiens B. 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 2011;301:H450–H458

26. Wei K, Jayaweera AR, Firoozan S, Linka A, Skyba DM, Kaul S. Quantification of myocardial blood flow with ultrasound-induced destruction of microbubbles administered as a constant venous infusion. Circulation 1998;97:473–483

27. Jeppesen J, Mogensen M, Prats C, Sahlin K, Madsen K, Kiens B. FAT/CD36 is localized in sarcolemma and in vesicle-like structures in subsarcolemma regions but not in mitochondria. J Lipid Res 2010;51:1504–1512
28. Lowry OH, Passonneau JV. *A Flexible System of Enzymatic Analysis*. New York, Academic Press, 1972.
29. Thomas JA, Schlender KK, Larner J. A rapid filter paper assay for UDP-glucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-14C-glucose. Anal Biochem 1968;25:486–499
30. Sjøberg KA, Holst JJ, Rattigan S, Richter EA, Kiens B. GLP-1 increases microvascular recruitment but not glucose uptake in human and rat skeletal muscle. Am J Physiol Endocrinol Metab 2014;306:E355–E362
31. Funai K, Schweitzer GG, Castorena CM, Kanzaki M, Cartee GD. In vivo exercise followed by in vitro contraction additively elevates subsequent insulin-stimulated glucose transport by rat skeletal muscle. Am J Physiol Endocrinol Metab 2010;298:E999–E1010
32. Hamada T, Arias EB, Cartee GD. Increased submaximal insulin-stimulated glucose uptake in mouse skeletal muscle after treadmill exercise. J Appl Physiol (1985) 2006;101:1368–1376
33. Wojtaszewski JF, Hansen BF, Kiens B, Richter EA. Insulin signaling in human skeletal muscle: time course and effect of exercise. Diabetes 1997;46:1775–1781
34. Cartee GD. Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle. Diabetologia 2015;58:19–30
35. Baron AD, Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G. Insulin-mediated skeletal muscle vasodilation contributes to both insulin sensitivity and responsiveness in lean humans. J Clin Invest 1995;96:786–792
36. Høeg LD, Sjøberg KA, Jeppesen J, et al. Lipid-induced insulin resistance affects women less than men and is not accompanied by inflammation or impaired proximal insulin signaling. Diabetes 2011;60:64–73
37. Mahmoud AM, Brown MD, Phillips SA, Haus JM. Skeletal muscle vascular function: a counterbalance of insulin action. Microcirculation 2015;22:327–347