Muscle Contraction, but not Insulin, Increases Microvascular Blood Volume (MBV) in the Presence of Free Fatty Acid-Induced Insulin Resistance

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Objective: Insulin and contraction each increase muscle microvascular blood volume (MBV) and glucose uptake. Inhibiting nitric oxide (NO) synthase blocks insulin’s but not contraction’s effects. We examined whether contraction could augment the MBV increase seen with physiologic hyperinsulinemia, and whether free fatty acid (FFA)-induced insulin resistance, differentially affects contraction vs. insulin-mediated increases in MBV.

Research design and Methods: Rats were fasted overnight. Plasma FFAs were increased by Intralipid/heparin infusion (3h), insulin was increased with a euglycemic clamp (3mU/min/kg), and hindlimb muscle contraction was electrically stimulated. Muscle MBV was measured using contrast-enhanced ultrasound (CEU). Insulin transport into muscle was measured using \(^{125}\text{I}\)-insulin. BQ-123 (0.4 mg/h) was used to block the endothelin-1 (ET-1) receptor A (ET\(_A\)).

Results: Superimposing contraction on physiologic hyperinsulinemia increased MBV within 10 min by 37% and 67% for 0.1 or 1 Hz, respectively (\(P<0.01\)). FFA-elevation alone did not affect MBV while 0.1 Hz stimulation doubled MBV (\(P<0.05\)) and increased muscle insulin uptake (\(P<0.05\)) despite high FFA. Physiologic hyperinsulinemia during FFA-elevation paradoxically decreased MBV (\(P<0.05\)). This MBV decrease was reversed by either 0.1 Hz contraction or ET\(_A\) antagonism and the combination raised MBV above basal.

Conclusions: Contraction recruits microvasculature beyond that seen with physiologic hyperinsulinemia by a distinct mechanism that is not blocked by FFA-induced vascular insulin resistance. The paradoxical MBV decline seen with insulin+FFA may result from differential inhibition of insulin-stimulated NO-dependent vasodilation relative to ET-1 vasoconstriction. Our results implicate ET-1 as a potential mediator of FFA-induced vascular insulin resistance.
Insulin delivery to muscle interstitium is reported to be rate-limiting for overall muscle insulin action (1, 2). Insulin promotes its own access to muscle interstitium by increasing blood flow (3), by recruiting microvasculature (4, 5) to expand the endothelial transporting surface available, and perhaps by also stimulating its own endothelial transport (6). Insulin's entry to muscle interstitium is delayed in insulin resistant states (7). This implicates insulin's vascular actions as a significant regulator of overall insulin action in muscle.

Elevated plasma concentrations of FFAs, as occur with obesity and type 2 diabetes, increase cellular lipid concentrations and are associated with insulin resistance in skeletal muscle, liver, and fat (8, 9). Experimentally, increased dietary fat (10-12) or acute infusion of a lipid emulsion induces insulin resistance (13-16). Increased intramyocellular lipid content in the context of obesity and DM2 could be one factor that contributes to muscle insulin resistance. Postprandially or in response to a euglycemic-insulin clamp, plasma [FFA] falls in insulin-sensitive individuals (17-19). This response is impaired in states of insulin resistance (8, 17, 19, 20).

Insulin also increases muscle blood flow and recruits microvasculature in both humans (21-24) and animals (4, 25-27), and both processes are inhibited by NOS blockade (27). Raising plasma FFAs initiates hemodynamic effects that include decreased compliance, increased blood pressure and heart rate, and increased vascular resistance (28-31). Raising plasma [FFA] blunts insulin’s NOS-dependent effects to mediate increases in both muscle microvascular blood volume (MBV) and glucose uptake (14, 32, 33). Thus, FFAs exert acute vascular as well as metabolic actions.

Insulin (34) and muscle contraction can each increase MBV and total flow in skeletal muscle (35-37). In addition, Wheatley et al. (38) observed that in the Zucker rat, insulin-mediated increases in MBV are blunted, but contraction-induced increases in MBV persisted. This suggests that exercise might recruit microvasculature via a mechanism that is distinct from that of insulin. Supporting this, we have recently shown that, like insulin, brief low-frequency isometric contraction of the rat hindlimb (0.1 Hz, 10 min) robustly increases MBV without any observed increase in total femoral blood flow (FBF) and, unlike insulin’s effect, this process is NO-independent (39).

In this study we addressed whether: (1) low-frequency contraction enhances muscle MBV and 3H-2-deoxyglucose (3H-2-DG) uptake beyond the effect of physiologic hyperinsulinemia; (2) lipid infusion differentially affects contraction vs insulin-mediated increases in MBV; and (3) lipid infusion blunts combined insulin- and contraction-mediated effects on MBV.

RESEARCH DESIGN AND METHODS

Animals – Male Sprague-Dawley rats (225–300 g) were obtained from Charles River Laboratories (Wilmington, MA), housed at 22 ± 2°C with a 12:12 h light:dark cycle and allowed free access to water and standard chow diet. All rats were fasted overnight prior to study. The experimental protocols were approved by the Animal Care and Use Committee of the University of Virginia.

Surgical Procedures – Animals were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital (50-55 mg/kg body weight) and placed on a surgical platform. Body temperature was maintained at 38°C with a heating lamp and pad. Both jugular veins and the carotid artery were cannulated with polyethylene tubing (PE-50) and used for intravenous infusions, arterial blood sampling and monitoring of mean
arterial blood pressure. A tracheostomy was performed to facilitate respiration. Animals were maintained under anesthesia for the duration of the experiment by IV infusion of aqueous sodium pentobarbital solution (0.6 mg/min⁻¹/kg⁻¹) via the carotid artery. For experiments in which total femoral blood flow was measured, the femoral vessels in the right hindlimb were exposed as described previously (23, 38) and an ultrasound flow probe (VB series 0.5 mm; Transonic Systems) was positioned over the femoral artery. The flow probe was interfaced through a flow meter to a personal computer. Femoral blood flow and arterial blood pressure were continuously acquired using Windaq software (Dataq Instruments Inc., Akron, OH). The animals were allowed 30 – 45 min to stabilize following surgical procedures prior to beginning experimental protocols.

Electrical Stimulation – Two metal electrodes were placed in the adductor tendons of the animal’s right hindlimb and the limb was secured in place. The left hindlimb served as the sham control. Muscles were isometrically contracted for 10 min (2 V, 0.5 ms) at a frequency given in each protocol (Grass S88 Pulse Generator; Astro-Med, West Warwick, RI). We previously reported that 0.1 Hz stimulation can increase in basal hindlimb MBV ~ 2-fold (39) without an increase femoral artery flow.

Contrast Enhanced Ultrasound (CEU) – Microvascular blood volume (MBV) was measured at the time points indicated in the protocols described in Fig. 1. Where indicated, animals received an infusion of phosphatidylcholine/PEG stearate coated decafluorobutane-filled microbubbles manufactured at the University of Virginia as reported previously (40). Microbubbles were diluted 1:3 in deoxygenated saline and infused at a rate of 10-15 µL/min for 11 min. Pulse-inversion ultrasound (HDI-5000, Philips Ultrasound) images of the proximal adductor muscle group (adductor magnus and semimembranosus) were obtained as previously described (5, 27) during the last 6 min at the time points specified in Fig 1. Data was analyzed using a commercial software package (Q-lab, Philips Ultrasound).

Experimental Protocols – Rats were studied in each of 6 experimental protocols (Fig. 1): (1) a 90 min euglycemic insulin clamp given as a primed (6 mU/kg/min x 8 min) continuous (3 mU/kg/min x 82 min) insulin infusion with a 30% glucose solution infused to maintain fasting euglycemia. During the last 30 min of the insulin clamp, 3 x 10 min periods of electrical stimulation (0.1, 1.0, 2.0 Hz) were imposed; (2) animals received a 70 min euglycemic insulin clamp (6 mU/kg/min x 8 min, then 3 mU/kg/min x 62 min) with a 50-µCi bolus of ³H-2-deoxyglucose (³H-2DG) (specific activity 48.0 Ci/mmol) given at 60 min followed by 0.1 Hz electrical stimulation of the right leg for the last 10 min; (3) animals were given a 3 hour infusion of 10ul/min of Intralipid/heparin (3.3% and 30 U/ml, respectively) together with a saline infusion over the last 2h (25 µL/min); (4) the same Intralipid/heparin infusion as in protocol 3 with a euglycemic insulin clamp (see protocol 1) infusion over the last 2h; (5) the same infusion protocol as in 4, with the addition of a continuous infusion of the ETA receptor antagonist, BQ-123 (0.4 mg/h x 3h) as reported previously (41); (6) Intralipid/heparin +saline as in protocol 3 with ¹²⁵I-insulin given half-way through the electrical stimulation period. Total femoral blood flow was measured in separate groups of rats (n=3-5) receiving infusion protocols 3 – 5.

³H-2-deoxyglucose Uptake Assay – Frozen muscles harvested from the stimulated and contralateral leg at the end of protocol 2 (Fig 1) were ground under liquid nitrogen and ~100 mg of tissue was homogenized in 2 mL of deionized water for 20 s on ice and spun for 10 min at 0°C (13,000 rpm). Free and
phosphorylated $^3$H-2-deoxyglucose ($^3$H-2-DG) in ~1.5 mL supernatant were separated using anion exchange chromatography (AG1-X8 resin) as previously described (14). $^3$H-2-DG-P was counted in a liquid scintillation system and from the total counts in the supernatant and plasma specific activity, muscle uptake was calculated ($\mu$g glucose/g wt tissue/min).

**Muscle $^{125}$I-insulin Content** – Low-frequency stimulation (0.1 Hz, 2 V, 0.5 ms) began 10 min before the end of the experiment (protocol 6). Five min after starting the electrical stimulation, the rats were given a 1.5 $\mu$Ci bolus of $^{125}$I-insulin. This mono-iodinated species binds to the insulin receptor similarly to native insulin and the tracer amount infused does not decrease systemic glucose concentrations. Gastrocnemius, soleus, and gracilis muscles were dissected from the stimulated and the control contra-lateral hindlimb. Protein bound $^{125}$I-iodine in blood and muscle samples was precipitated using 10% trichloroacetic acid and radioactivity was measured in a gamma counter and expressed as dpm per gram of dry tissue. This was further normalized to plasma specific activity.

**Plasma Insulin and Endothelin-1 Concentration** – Blood samples were taken at the time points indicated in each protocol (Fig. 1), quickly spun down and plasma was kept frozen (-80 °C). The insulin concentration in plasma samples was run in duplicate using a radioimmunoassay (LINCO Research, St. Charles, MI). ET-1 concentration in samples was measured in duplicate using an enzyme-linked immunoassay (ALPCO Diagnostics, Winham, NH).

**Western Detection of AMPK Phosphorylation**– Muscle samples were rapidly frozen and subsequently prepared for polyacrylamide gel electrophoresis and Western blotting as previously described (5). Proteins were transferred to nitrocellulose membranes and detected using antibodies against AMPK$\alpha$ and p-AMPK$\alpha$ (Thr$^{172}$) according to manufacturers’ protocols. Membranes were incubated with IgG-peroxidase linked secondary antibody for 60 min. Bands were detected via chemiluminescence using ECL reagents and quantified by ImageQuant 3.3 software.

**Chemicals and Reagents** – Intralipid 20% (Baxter Healthcare, Deerfield, IL), endothelin receptor A (ET$_A$) antagonist BQ-123 (Alexis Biochemicals, San Diego, CA); $^3$H-2-deoxyglucose (Amersham Biosciences, UK); $^{125}$I-insulin (Perkin Elmer, Waltham, MA); AG-1X8 anion exchange resin (Bio-Rad Laboratories, Hercules, CA); AMPK antibodies (Cell Signaling Technology, Inc., Beverly, MA); ECL reagents (Amersham Biosciences, UK).

**Statistical Analysis** – Statistical calculations were performed using SigmaStat® software. Individual tests are described in the figure legends. Data are presented as means ±SEM.

**RESULTS**

**Contraction further increases MBV after insulin-induced microvascular recruitment.** In agreement with previous studies (4, 5), 60 min of insulin increased MBV by ~65 % compared to baseline (Fig. 2). We previously reported, that doses of insulin above the 3 mU/min/kg dose used here further enhanced MBV only minimally (34). Isometric contraction for 10 min at 0.1 Hz significantly ($P < 0.05$) increased MBV by an additional ~37% over 60 min of insulin alone. Stimulation at 1 or 2 Hz further increased MBV. The increases seen at 1 and 2 Hz cannot simply be attributed to the longer duration of stimulation, as (in separate experiments) we observed that 45 min of continuous stimulation at 0.1 Hz does not increase MBV beyond that seen during the first 10 min of stimulation when (data not shown). We previously reported (39) that
contraction alone (in the absence infused insulin) increased MBV by 131%, 200%, and 200% above basal at frequencies of 0.1, 1.0, and 2.0 Hz, respectively. These incremental increases above basal were similar to those seen in the current study (100%, 176%, and 176% above basal for these same frequencies in the setting of hyperinsulinemia, Fig. 2). These findings demonstrate that contraction increases MBV beyond that observed at insulin concentrations that are physiological and near maximally effective in recruiting the microvasculature (34).

**Low-frequency contraction further enhances insulin-stimulated 3H-2DG uptake in muscle.** Study protocol 2 addressed whether very low-frequency (0.1 Hz) electrical stimulation of brief (0.5 msec) duration, which would be expected to minimally effect leg energy expenditure (but which effectively enhances microvascular blood volume) affects muscle glucose disposal during a euglycemic clamp. We first observed that in the absence of a systemic insulin infusion, this brief contractile stimulus did not affect basal muscle 2-deoxyglucose uptake (2.7 ± 0.3 vs. 2.0 ± 0.2 ug glucose/g wt tissue/min for 0.1 Hz and control legs, respectively (Fig. 3B). At the end of the euglycemic clamp muscle 2-deoxyglucose uptake was significantly stimulated compared to saline controls and glucose uptake by the electrically stimulated leg exceeded that in the control contralateral limb by ~1.3-fold (Fig. 3B; P < 0.05).

We also examined whether 0.1 Hz stimulation enhanced muscle AMPKα phosphorylation as this is often correlated with exercise stimulated glucose uptake and metabolism in muscle. In rats receiving only a saline infusion, neither 10 min nor an extended 45 min of 0.1 Hz contraction significantly increased AMPKα phosphorylation as compared to control (Fig. 3A). In contrast, as a positive control, 10 min of high-frequency electrical stimulation at 8.0 Hz significantly increased AMPKα phosphorylation.

**FFAs do not affect basal or contraction-mediated increases in MBV nor prevent contraction-mediated 125I-insulin uptake.** We previously reported that total femoral blood flow (FBF) remains unchanged in response to 10 min of 0.1 Hz contraction, while skeletal muscle insulin uptake is modestly increased by this brief 0.1 Hz contractile stimulus (39). In the present study, plasma FFA levels increased ~1.5-fold over baseline after 1 and 3 h of the infusion protocol (Table 1). With lipid infusion, total FBF trended towards a slight increase after 3 h but this was not significant (Fig. 4B). When insulin was added to the FFA infusion, femoral blood flow declined significantly. Presumably this was secondary to increased tone of resistance vessels in the leg. Others had reported a paradoxic vasoconstriction induced by insulin when FFAs were infused (42). Infusing BQ 123 for one hour before and throughout the insulin infusion did not prevent this decline (Fig 4B).

In response to Intralipid/heparin alone, MBV remained unchanged after either 1 or 3 h of infusion (Fig. 4A). However, MBV increased ~1.8-fold within 10 min of initiating 0.1 Hz contraction (vs. 1h and 3h; P < 0.05). Here again, in the context of FFA-provoked insulin resistance 0.1 Hz contraction still significantly promoted the delivery of 125I-insulin to muscle as compared to the resting control limb (Fig. 5). We had previously observed a similar effect when saline was infused instead of FFA (39).

**Contraction, but not insulin, increases MBV in the presence of FFAs.** Infusion of Intralipid/heparin (Protocol 3) decreased the glucose infusion rate required to maintain euglycemia during the insulin clamp indicating insulin resistance (Table 1). Interestingly, MBV did not increase but declined slightly during the first 30 min after the onset of insulin infusion, and was
significantly below baseline after 2 h (Fig. 6; P < 0.05). This was paralleled by a gradual and significant decrease in total FBF over the last 60 min studied in a separate group of 5 animals (Fig. 4B; P < 0.05). At the end of the 3 h of Intralipid/heparin and 2 h of insulin, 0.1 Hz muscle contraction increased MBV by >3-fold within 10 min, reaching levels comparable to but not significantly above baseline.

**ETA receptor antagonism prevents the effect of combined lipid and insulin to decrease MBV.** In addition to stimulating NO production, insulin can enhance production of the vasoconstrictor ET-1 via the ERK1/2 protein kinase cascade. To address whether the unexpected MBV decrease produced by combined Intralipid/heparin and insulin infusion might be caused by increased ET-1 production, we measured plasma ET-1 concentrations at baseline and at the end of Protocol 4. The circulating ET-1 concentrations did not change during the infusion Protocol (Table 1). As ET-1 is thought to act principally in a paracrine fashion we investigated this further by infusing the ETA receptor antagonist, BQ-123 (0.4 mg/h) (41) concomitantly with Intralipid/heparin throughout the duration of the experiment. In this protocol, MBV did not change significantly during combined BQ-123, Intralipid/heparin, and insulin infusion (mean 14% rise, p=NS), unlike the 32% decline observed in MBV in protocol 4, without BQ-123 present (Fig. 6). When the change in MBV was compared between animals studied using protocols 4 and 5, the MBV decline in protocol 4 was of borderline (p=0.07) significance. This pattern of MBV response contrasts with the lack of effect of BQ 123 on the insulin-induced decline in femoral blood flow (Fig 4B) noted previously. However, low frequency contraction at 0.1 Hz (10 min) significantly increased MBV in the BQ-123, Intralipid/heparin, and insulin group.

**DISCUSSION**

In the present study, we confirmed that insulin alone increased the skeletal muscle microvascular volume accessible to microbubbles significantly over basal values. However, when compared to basal (pre-insulin or contraction) the combination of insulin + exercise had no greater effect on MBV versus contraction alone at either 0.1, 1.0, or 2.0 Hz. Muscle contraction did increase MBV above that seen with insulin alone. As the insulin infusion rate used here exerts near maximal effects on MBV, the significant augmentation following contraction suggest that contraction is a more potent stimulus to microvascular recruitment. We had previously found that insulin recruits microvasculature by a NO-dependent (27) and exercise by a NO-independent mechanism (39). The additional finding that FFA elevation blocked insulin’s but not contraction’s ability to recruit microvasculature further differentiates these several mechanisms that regulate capillary recruitment.

Low-frequency muscle contraction (0.1 Hz) also significantly increased muscle 2-deoxyglucose uptake during hyperinsulinemia but not when insulin concentrations remained at post-absorptive levels. In addition, it did not affect AMPKα phosphorylation. Lack of any increase of 2-deoxyglucose uptake under conditions of basal insulin underscores the very light workload from this contraction stimulus protocol. It appears likely that the increased muscle 2-deoxyglucose uptake during insulin infusion (euglycemic clamp) + muscle contraction could be explained by the observed changes in MBV. Such changes would increase endothelial surface available for insulin delivery to muscle interstitium, which in turn may have enhanced insulin’s action to stimulate muscle glucose uptake. Indeed, we had previously reported that a 0.1 Hz contraction significantly increased the muscle
uptake of iodinated insulin (39). In the current study we observed that FFA-provoked insulin resistance did not block the effect of 0.1 Hz contraction to increase \(^{125}\)I-insulin delivery to muscle. These findings raise the possibility that muscle contraction induced increases in MBV and enhanced insulin delivery to muscle interstitium may be one of the mechanisms by which exercise ameliorates insulin resistance.

We also wanted to ascertain the effect of acute infusion of Intralipid/heparin on contraction- and insulin-mediated increases on muscle MBV. Contraction appeared to increase MBV normally during Intralipid/heparin infusion. In contrast, infusing insulin with Intralipid/heparin paradoxically decreased MBV below baseline. Adding low-frequency muscle contraction restored MBV to baseline values but did not produce increases over baseline as was seen when either insulin or FFA alone were followed by electrical stimulation.

Insulin stimulates the release of both ET-1 and NO by endothelial cells. Inhibiting PI-3-kinase reduces NO-mediated arteriolar dilation but enhances constriction by ET-1 (42). Insulin resistant individuals have higher fasting ET-1 levels than healthy controls. Acute increases in both plasma insulin and FFAs in normal subjects raises plasma endothelin-1 to concentrations seen with insulin resistance. (43). Interestingly, it was recently shown that ET\(_A\) receptor antagonism in obese insulin-resistant individuals increased skeletal muscle hemodynamic and metabolic responses to insulin (44). We hypothesized that the balance between insulin's effect to increase NO (vasodilation) and ET-1 (vasoconstriction) might be affected in the context of elevated plasma FFAs.

We were not able to detect an effect of elevated plasma FFA concentrations on plasma ET-1 concentration. However, antagonism of ET\(_A\) receptors with BQ-123 prevented the decline in MBV observed in response to combined FFAs and insulin. This suggests that FFAs might enhance insulin-mediated ET-1 release. Although BQ-123 prevented MBV from decreasing in response to combined FFA elevation and insulin, MBV did not rise above baseline values. Adding a contraction stimulus further increased MBV. Therefore, antagonism of ET\(_A\) alone might not be expected to fully restore the vascular response to insulin in the presence of FFAs. Another possible mechanism that might explain these results is that FFAs might facilitate conversion of NO to reactive oxygen species as has been previously suggested (45, 46), and in the context of elevated plasma FFAs, insulin-mediated NO production might lead to biologically unfavorable effects.

We conclude that in the absence of elevated plasma FFAs, insulin and low-frequency muscle contraction can each increase MBV. Muscle contraction stimulates muscle insulin uptake and this contributes to the enhancing effect of contraction on glucose metabolism. Raising plasma FFA concentration does not block the effect of contraction to recruit microvasculature. However, raising FFA alters endothelial function to provoke a paradoxical insulin-mediated de-or recruitment of the microvasculature. The effect of BQ123 to block MBV decreases triggered by combining hyperinsulinemia with elevated plasma FFA suggests a shift in a balance of insulin action on the microvascular endothelium towards increased ET-1 production at least partly underlies this paradoxical vasoconstrictr response. However, this does not exclude the possibility that in the context of elevated plasma FFAs, insulin signaling in the vascular endothelium might activate other hemodynamically unfavorable actions. The fact that BQ123 did not also prevent the decline in leg blood flow (Fig 4B), may also suggest either other pathways by which insulin may cause vasoconstriction or that resistance vessels are not as sensitive to
BQ123 and the dosages used here. These findings extend our understanding of the distinct mechanisms used by insulin and exercise to act on the vasculature within skeletal muscle.
Table 1. Physiological parameters
Data are presented as means ± SEM (n = 6 – 9 per group). *P < 0.05 vs. Insulin; **P < 0.05 vs. baseline; #P < 0.05 vs. baseline (One-way ANOVA). Blood glucose (BG), glucose infusion rate (GIR), free fatty acids (FFAs), Insulin (Ins), BQ-123.

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<td>FFAs 3 h + 0.1 Hz</td>
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<td>FFAs 1.5 h + Ins 30 min</td>
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**Figure Legends**

**Figure 1. Lipid and insulin infusion protocols**
Experimental Protocols. Time-points for microbubble infusion and data acquisition are denoted by (*) for contrast enhanced ultrasound (CEU). Blood samples (20 uL) were taken at (t = 0, 30, 60, 120, 180).

**Figure 2. Combined effect of insulin and contraction on MBV**
Protocol 1 results (n = 7): Effect of insulin (3 mU/kg/min x 90 min) and isometric hindlimb contraction (0.1, 1.0, 2.0 Hz; 10 min/bout) on microvascular blood volume (MBV). *P < 0.05 vs. baseline (white bar); **P < 0.05 vs. baseline, Ins 60 min (light gray); #P < 0.05 vs. baseline, Ins 60 min and 0.1 Hz (dark gray); One-way RM ANOVA.

**Figure 3. Effect of contraction on AMPKα phosphorylation and glucose uptake**
A. Effect of isometric contraction on fasting basal muscle AMPKα phosphorylation (Thr172). *P < 0.05 vs. 0.1 Hz (10 min), 0.1 Hz (45 min); One-way ANOVA (n = 5). B. Protocol 2 results: Combined effect of insulin and brief, low-frequency contraction on muscle glucose utilization (n = 6). A 3H-2-deoxyglucose (3H-2DG) bolus (50 µCi) was given right before the onset of contraction and circulated a total of 10 min. *P < 0.05 vs. basal conditions, #P < 0.05 vs. Insulin + Rest, paired t-test.

**Figure 4. Effect of FFAs and contraction on MBV and FBF**
A. Protocol 3 results: The effect of FFA-provoked insulin resistance on microvascular blood volume (MBV) after 1h, 3h and 3h + 10 min 0.1 Hz isometric hindlimb muscle contraction (n = 6). *P < 0.05 vs. FFAs 1h, FFAs 3h; One-way RM ANOVA. B. Effects of treatment on femoral blood flow (FBF); Protocols 3-5 (n = 4 per group). *P < 0.05 One-way RM ANOVA (vs. baseline).

**Figure 5. Effect of FFAs on contraction-mediated muscle 125I-insulin uptake.**
An I.V. 1.5 µCi bolus of iodinated insulin (125I-insulin) was given 5 min after starting low-frequency (0.1 Hz) isometric contraction (Protocol 2). Intact insulin in wet tissue was determined using TCA precipitation and then normalized to dry tissue weight (n = 7).

**Figure 6. Effect of FFAs microvascular responses to insulin and contraction**
Intralipid/heparin (FFAs) and insulin (Ins) were infused as described in experimental Protocol 4. In the presence of elevated FFAs, insulin had a paradoxical effect to decrease MBV vs. baseline. Brief low-frequency contraction (0.1 Hz, 10 min) restored MBV to baseline (n = 9). *P < 0.05 vs. all other treatment groups; One-way RM ANOVA.

**Figure 7. Combined ETα antagonism and FFA elevation on the microvascular response to insulin and contraction.**
The endothelin A (ETα) receptor agonist, BQ-123 (0.4 mg/h) was infused with Intralipid/heparin (FFAs) as described in Protocol 5. The physiologic euglycemic-hyperinsulinemic clamp (3 mU/kg/min x 90 min) started 1h after FFAs/BQ infusion. Contraction (0.1 Hz) was superimposed over the last 10 min. MBV was measured at the indicated timepoints (n = 6). *P < 0.05 vs. all other groups; One-way RM ANOVA.
REFERENCES


30. **Steinberg HO, Baron AD** 2002 Vascular function, insulin resistance and fatty acids. Diabetologia 45:623-634.


Figure 1

PROTOCOL 1
(n = 7)

PROTOCOL 2
(n = 6)

PROTOCOL 3
(n = 6)

PROTOCOL 4
(n = 9)

PROTOCOL 5
(n = 6)

PROTOCOL 6
(n = 7)

*Contrast Enhanced Ultrasound (CEU)*

0  30  60  90  120  150  180

Time (min)
Figure 2

The graph illustrates MBV (Acoustic Intensity, AI) for different conditions: baseline, insulin 1h, 0.1 Hz, 1.0 Hz, and 2.0 Hz. The data shows a significant increase in MBV at 0.1 Hz, marked with an asterisk (*). Additionally, the 1.0 Hz and 2.0 Hz conditions are marked with a hash (#), indicating a different statistical significance or comparison.
Figure 3

A.  

AMPK\(\alpha\) phosphorylation (Thr172) (Normalized to Total AMPK\(\alpha\))

- Rest (control)
- 0.1 Hz 10 min
- 0.1 Hz 45 min
- 8.0 Hz 10 min

B.  

Glucose Uptake (\(\mu\)g glucose/g muscle/min)

- Basal Resting (Control)
- Basal 0.1 Hz (10 min)
- Insulin + Rest (Control)
- Insulin + 0.1 Hz (10 min)
Figure 4

A. MBV (Acoustic Intensity: AI)

B. FBF (mL/min)
Figure 5

![Graph showing intact 125I-insulin uptake (dpm/g dry tissue) with bars for FFAs and FFAs + 0.1 Hz. The graph indicates a significant difference (*) between the two conditions.]
Figure 6
Figure 7

![Graph showing MBV (Acoustic Intensity, AI) across different conditions.](image)