



Liraglutide and Exercise Synergistically Attenuate Vascular Inflammation and Enhance Metabolic Insulin Action in Early Diet-Induced Obesity

Jia Liu, Kevin W. Aylor, and Zhenqi Liu

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Inflammation-induced vascular insulin resistance is an early event in diet-induced obesity and contributes to metabolic insulin resistance. To examine whether exercise and glucagon-like peptide 1 (GLP-1) receptor agonism, alone or in combination, modulate vascular and metabolic insulin actions during obesity development, we performed a euglycemic insulin clamp in adult male rats after 2 weeks of high-fat diet feeding with either access to a running wheel (exercise), liraglutide, or both. Rats exhibited increased visceral adiposity and blunted microvascular and metabolic insulin responses. Exercise and liraglutide alone each improved muscle insulin sensitivity, but their combination fully restored insulin-mediated glucose disposal rates. The combined exercise and liraglutide intervention enhanced insulin-mediated muscle microvascular perfusion, reduced perivascular macrophage accumulation and superoxide production in the muscle, attenuated blood vessel inflammation, and improved endothelial function, along with increasing endothelial nucleus translocation of NRF2 and increasing endothelial AMPK phosphorylation. We conclude that exercise and liraglutide synergistically enhance the metabolic actions of insulin and reduce vascular oxidative stress and inflammation in the early stage of obesity development. Our data suggest that early combination use of exercise and GLP-1 receptor agonism might be an effective strategy in preventing vascular and metabolic insulin resistance and associated complications during the development of obesity.

Muscle microvasculature critically regulates insulin delivery to and action in muscle and is also an important insulin action site (1–3). Insulin dilates the precapillary arterioles to increase muscle microvascular perfusion and facilitate its

ARTICLE HIGHLIGHTS

- Inflammation-induced vascular insulin resistance occurs early in diet-induced obesity and contributes to metabolic insulin resistance.
- We examined whether exercise and GLP-1 receptor agonism, alone or in combination, modulate vascular and metabolic insulin actions during obesity development.
- We found that exercise and liraglutide synergistically enhanced the metabolic actions of insulin and reduced perimicrovascular macrophage accumulation, vascular oxidative stress, and inflammation in the early stage of obesity development.
- Our data suggest that early combination use of exercise and a GLP-1 receptor agonist might be an effective strategy in preventing vascular and metabolic insulin resistance and associated complications during the development of obesity.

own delivery to the muscle interstitium via the nitric oxide (NO) signaling pathway (4). This feed-forward system that couples the vascular and metabolic actions of insulin is blunted in insulin-resistant states, which contributes to the development of metabolic insulin resistance (5). NO is critically important in this process because inhibiting NO production abolishes the microvascular actions of insulin and decreases insulin-mediated glucose disposal by up to 40% (6). This is physiologically important because muscle is a major insulin action target and responsible for ~80–90% of insulin-stimulated glucose disposal during insulin infusion (7).

Division of Endocrinology and Metabolism, Department of Medicine, University of Virginia Health System, Charlottesville, VA

Corresponding author: Zhenqi Liu, zl3e@virginia.edu

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Lifestyle modification is the cornerstone of diabetes prevention and management. Although exercise exerts insulin sensitization actions via multiple mechanisms, muscle microvasculature plays an important role in regulating exercise-induced insulin sensitization (8). Exercise is the most potent physiological factor that increases muscle microvascular perfusion. Even simple handgrip markedly increases cardiac (9) as well as skeletal muscle (10) microvascular perfusion in healthy humans. In rodents, low-frequency contraction recruits muscle microvasculature and facilitates muscle insulin delivery in the absence of total tissue blood flow increases (11). Muscle contraction also increases the expression and secretion of the vascular endothelial growth factor (VEGF) family of proteins, which acts on the capillary endothelial VEGF receptors (VEGFRs) to stimulate angiogenesis (12). Importantly, the microvascular actions of exercise in muscle are preserved in insulin-resistant states (13).

The vascular endothelium expresses abundant glucagon-like peptide 1 (GLP-1) receptors (14). In addition to its well-characterized glycemic actions, GLP-1 exerts beneficial actions on the vasculature (15). We have shown that acute GLP-1 infusion increases microvascular recruitment in human and rodent skeletal muscle (16–18), likely via protein kinase A-mediated endothelial NO synthase activation (16,19). This action is independent of insulin (16,17) and preserved in insulin-resistant states (20,21). Treatment of rodents with GLP-1 receptor agonist liraglutide for 4 weeks prevented high-fat diet (HFD)-induced microvascular insulin resistance and attenuated metabolic insulin resistance (22).

In the current study, we aimed to define whether exercise (with access to a running wheel) and GLP-1 receptor agonism, alone or in combination, would alter the microvascular and metabolic actions of insulin during insulin-resistance development and the potential underlying mechanisms.

RESEARCH DESIGN AND METHODS

Animal Preparation and Experimental Protocols

Adult male Sprague-Dawley rats (~175–200 g) were purchased from Charles River Laboratories (Wilmington, MA), individually housed at $22 \pm 2^\circ\text{C}$ on a 12-h light-dark cycle, and fed an HFD (60% calories from fat; 5.21 kcal/g; cat. no. D12492; Research Diets, Inc.) for 2 weeks after 1 week of acclimatization. Rats were randomly assigned to one of the following groups: 1) control group (sedentary), 2) liraglutide group (200 $\mu\text{g/kg}$ subcutaneously twice daily), 3) exercise group (with ad libitum access to a running wheel [Med Associates, Inc., St. Albans, VT] attached to the cage), or 4) liraglutide plus exercise group (200 $\mu\text{g/kg}$ subcutaneously twice daily plus ad libitum access to a running wheel). Rats in control and exercise groups received an equal amount of saline injection subcutaneously twice daily. Each rat was supplied a maximal food amount of 20 g per day with water access ad libitum. For cages with a running wheel attached, daily running distance was tracked and recorded automatically. At

the end of the 2-week intervention, rats were studied under one of the following two protocols.

In protocol 1, rats were fasted overnight and anesthetized with thiobutabarbital (130–150 mg/kg Inactin i.p.; Sigma-Aldrich). They were placed in a supine position on a heating pad to ensure euthermia and intubated to maintain a patent airway. The carotid artery and jugular vein were cannulated with PE-50 polyethylene tubing for arterial blood pressure monitoring, arterial blood sampling, and various infusions. After a 30- to 45-min period to ensure hemodynamic and anesthesia stability, each rat received a euglycemic hyperinsulinemic clamp (3 mU/kg/min) for 120 min, with arterial blood glucose determined every 10 min using an Accu-Chek Advantage glucometer (Roche Diagnostics). Dextrose (30% wt/vol) was infused at a variable rate to maintain blood glucose within 10% of basal, and the glucose infusion rate (GIR) and GIR area under the curve were calculated. Hindlimb muscle microvascular blood volume (MBV), microvascular blood flow (MBF) velocity (MFV), and MBF were determined using contrast-enhanced ultrasound, as described previously (23). MBV represents the volume of microvasculature being perfused and MFV the velocity of blood flow. MBF is the product of MBV and MFV. Serum insulin, free fatty acid (FFA), and NO levels were measured before and after insulin clamps, as described previously (22) and in the Supplementary Material. Rats were euthanized by anesthetic overdose at the end of the study. Hindlimb muscle, aortas, and epididymal fat pads were rapidly excised, freeze clamped, and stored at -80°C for later analysis.

In protocol 2, overnight-fasted rats were euthanized using CO_2 overdose. The aorta was quickly removed and used for endothelial cell isolation and immunofluorescence staining, as described below. Distal saphenous arteries were dissected and used for determination of endothelial function, as previously described (24–27).

Mean arterial pressure was monitored via a sensor connected to the carotid arterial catheter (Harvard Apparatus and AD Instruments) throughout the study. This investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (28). The study protocols were approved by the Animal Care and Use Committee of the University of Virginia.

Immunofluorescence Staining of Endothelial Cells

Freshly resected rat aortas were immediately cut into small pieces (~3 mm $\times$ 3 mm) and used for endothelial cell immunofluorescence staining of reactive oxygen species (ROS), nuclear factor erythroid 2-related factor 2 (NRF2), and phosphorylated AMPK and endothelial NO synthase (eNOS), as described (29). Cells were freshly transferred from the vessel wall to the coverslip before immunofluorescence staining.

Immunofluorescence Staining of Perimicrovessel Macrophage and Superoxide in Muscle

The gastrocnemius was rapidly fixed in 4% paraformaldehyde, transferred to 30% sucrose the following day until

tissue sank, and then embedded in an optimal cutting temperature compound (Tissue-Tek; Sakura Finetek USA, Inc.), frozen, and stored at -80°C . Cryosections were done transversely at 5–6 microns and stored in a freezer until use. For macrophage immunostaining, tissue sections were thawed, rehydrated in PBS, and permeabilized with 0.2% Triton X-100 at room temperature for 1 h. After rinsing in PBS and blocking with 10% donkey serum, the sections were incubated with primary antibody goat anti-mouse/rat CD31/PECAM-1 antibody in a humidified chamber overnight at 4°C , followed by secondary staining with Alexa Fluor Plus 647 donkey anti-goat secondary antibody at room temperature for 1 h. From this step forward, sections were protected from light. The sections were then rinsed in PBS and incubated with 10% goat serum at room temperature for 1 h, followed with a second primary staining using rabbit anti-mouse/rat CD68 antibody in a humidified chamber overnight at 4°C and secondary staining with Alexa Fluor 488 goat anti-rabbit secondary antibody at room temperature for 1 h. For detection of superoxide production in muscle, muscle cryosections were incubated in $2\text{ }\mu\text{mol/L}$ dihydroethidium solution (Thermo Fisher Scientific, Waltham, MA) for 1 h at room temperature protected from light.

After rinsing off excess reagent, the sections were covered with antifade mounting medium with DAPI and immediately imaged using a fluorescence microscope (Olympus Spinning Disk Confocal) at $\times 20$ magnification.

Nuclear Factor- κB DNA-Binding Activity Assay

Frozen aortas and gastrocnemius muscle were used for the determination of NF- κB activity, as previously described (25,30).

Determination of Protein Expression and Phosphorylation

Total eNOS, Akt (protein kinase B), extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), AMPK α , VEGF, VEGFR1, VEGFR2, β -actin, and phosphorylated eNOS (Ser¹¹⁷⁷), Akt (Ser⁴⁷³), ERK1/2 (Thr²⁰²/Tyr²⁰⁴), and AMPK α (Thr¹⁷²) were determined in aorta and gastrocnemius samples collected after insulin infusion using Western blot analysis (29).

Statistical Analysis

All data are presented as means $\pm$ SEMs. Statistical analyses were performed with GraphPad Prism 9 (San Diego, CA), using ANOVA with post hoc analysis or Student *t* test as appropriate. A *P* value of <0.05 was considered statistically significant.

Data and Resource Availability

The data sets generated or analyzed in this study can be obtained upon reasonable request from the corresponding author.

RESULTS

Animal Characteristics

Figure 1 shows animal characteristics after 2 weeks of HFD with or without intervention (liraglutide, exercise, or liraglutide plus exercise). Similar to our prior observations (29), rats receiving HFD alone consumed ~ 20 g food per day. Liraglutide or exercise alone significantly and similarly reduced the amount of food intake (Fig. 1A) and HFD-induced body weight gain (Fig. 1B). Both liraglutide and exercise resulted in lower visceral adiposity, but exercise seemed to be more effective (Fig. 1C). The combined intervention was much more effective than either liraglutide or exercise alone for reducing body adiposity and preventing body weight gain. Fasting blood glucose levels were not significantly different among all groups (Fig. 1D). Use of liraglutide did not alter either daily or mean running distance (Fig. 1E and F).

Effects of Liraglutide, Exercise, and Liraglutide Plus Exercise on Metabolic Insulin Response in HFD-Fed Rats

To assess the impact of the various interventions on metabolic insulin response, we performed a 2-h euglycemic hyperinsulinemic clamp in all animals at the end of the 2-week intervention. We previously showed that in chow-fed rats, the steady-state GIR was ~ 8 – 10 mg/kg/min (26,27,31), and 2 weeks of HFD decreased it to ~ 5 mg/kg/min (29). As shown in Fig. 2, HFD feeding similarly reduced insulin-stimulated whole-body glucose disposal, with a steady-state GIR of 5.8 ± 0.5 mg/kg/min. Liraglutide or exercise each increased insulin-stimulated glucose disposal by $\sim 17\%$ (liraglutide 6.8 ± 1.33 mg/kg/min; exercise 6.8 ± 0.73 mg/kg/min), which was not statistically significant. However, the liraglutide and exercise combination strikingly improved insulin-stimulated GIRs in HFD-fed rats to the levels seen in chow-fed rats. This improvement was evident within 30 min and lasted during the entire clamp course, with a higher steady-state GIR (10.3 ± 1.22 mg/kg/min; $P < 0.05$) $\sim 80\%$ higher compared with the HFD alone group (Fig. 2A–C).

Liraglutide alone modestly, and exercise alone or in combination with liraglutide significantly, lowered fasting serum insulin concentrations (Fig. 2D). Postclamp serum insulin concentrations were significantly lower in all intervention groups compared with HFD controls (Fig. 2E). The insulin sensitivity indices (M/I ratios) and the steady-state GIRs corrected by serum insulin concentrations were significantly higher in both liraglutide and exercise alone groups, with a further increase in the combination group (Fig. 2E). Although none of the interventions altered basal serum FFA levels, insulin infusion potentially reduced serum FFA levels in all groups (Fig. 2F).

Effects of Liraglutide, Exercise, and Liraglutide Plus Exercise on Microvascular Insulin Resistance

Given that insulin action in muscle microvasculature is closely coupled with the metabolic effects of insulin in muscle (32,33), we examined insulin-mediated microvascular perfusion. Consistent with our previous studies (21,22,25), insulin

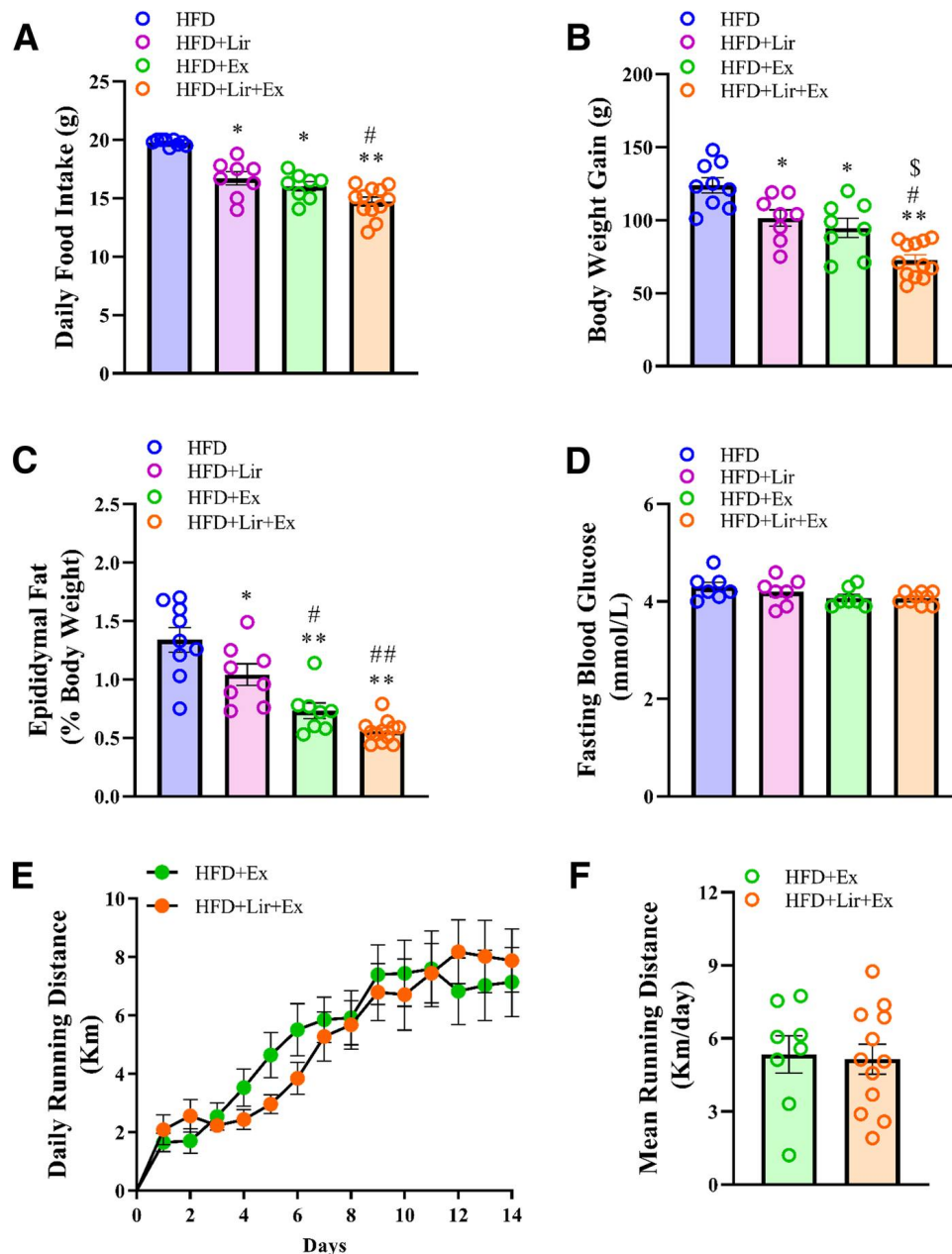


Figure 1—A–F: Animal characteristics: daily food intake (A), body weight gain (B), body adiposity (C), fasting blood glucose level (D), and daily (E) and mean (F) running distance of rats with access to running wheel ($n = 8$ – 12 per group). Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. HFD alone group; # $P < 0.05$, ## $P < 0.01$ vs. HFD plus liraglutide (Lir) group; \$ $P < 0.05$ vs. HFD plus exercise (Ex) group using ANOVA with Tukey post hoc analysis.

failed to induce muscle microvascular recruitment in HFD-fed rats, as indicated by a lack of increase in either MBV or MBF during insulin infusion (Fig. 3). Exercise, but not liraglutide, enabled a marked increase in insulin-mediated increases in muscle MBV and MBF without altering MFV, suggesting that exercise effectively rescued muscle microvascular insulin sensitivity in HFD-fed rats. Combined liraglutide and exercise intervention did not further increase microvascular insulin responses when compared with exercise alone (Fig. 3A–C). The increase in insulin-mediated muscle microvascular recruitment was paralleled by a significant increase in serum

NO levels (Fig. 3D) in the exercise alone and combination groups. We did not measure NO levels at all time points because we previously demonstrated robust parallel increases in plasma NO content and insulin-mediated microvascular recruitment (22).

Mean arterial pressure was comparable among all groups during the entire infusion period (Fig. 3E). When data from all groups were pooled together, insulin-mediated increases in muscle MBV correlated positively with steady-state GIRs ($P < 0.01$) (Fig. 3F). In addition, both insulin-mediated microvascular perfusion (at all points measured) and

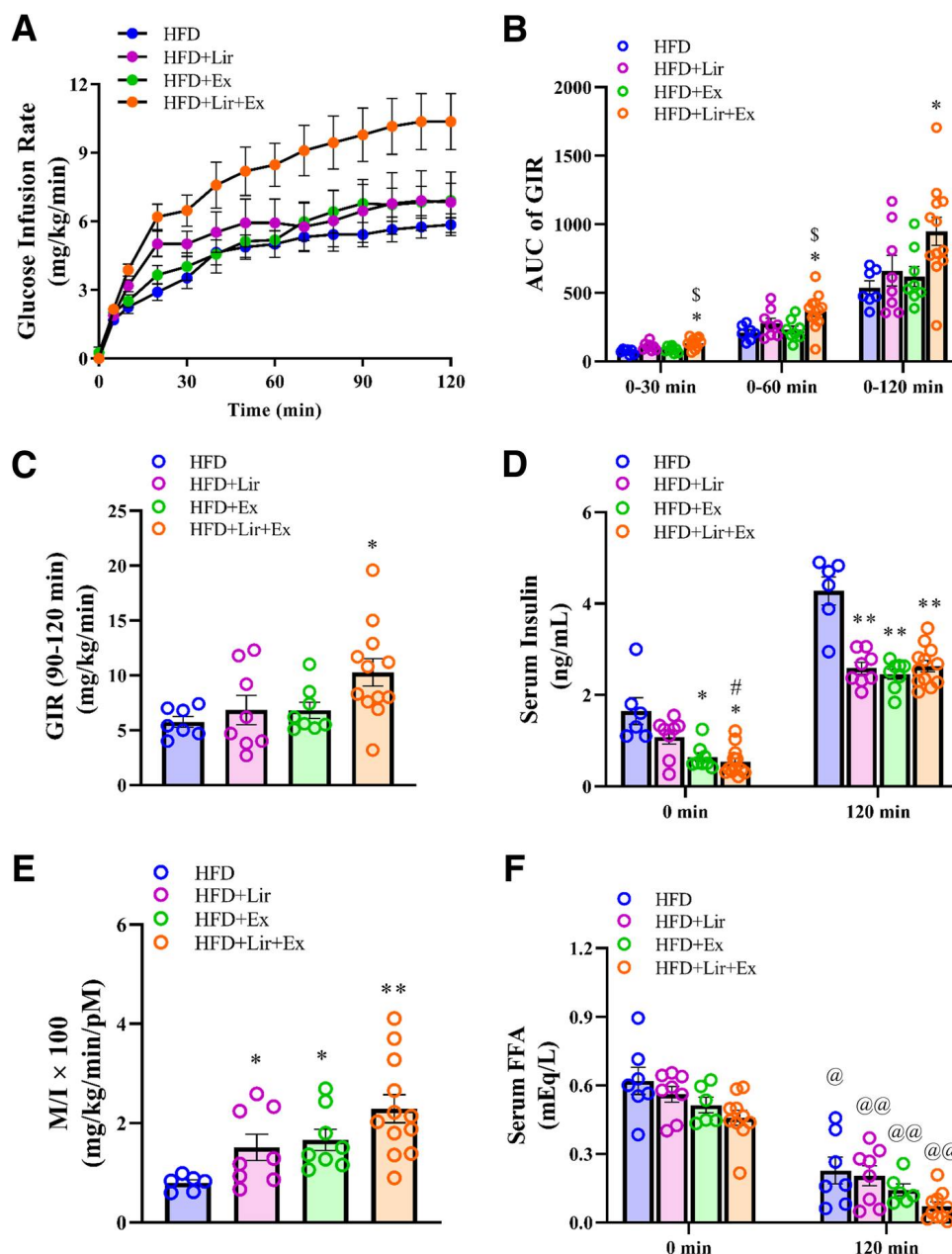


Figure 2—A–F: Effects of exercise, with or without liraglutide (Lir), on metabolic insulin responses: time course of GIRs (A), area under the curve (AUC) of GIRs (B), steady-state GIRs (C), serum insulin concentrations (D), insulin sensitivity indices (M/I ratios) (E), and serum FFA concentrations (F) ($n = 6$ –12 per group). Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. HFD alone group; # $P < 0.05$ vs. HFD plus Lir group; \$ $P < 0.05$ vs. HFD plus exercise (Ex) group using ANOVA with Tukey post hoc analysis. @ $P < 0.001$, @@ $P < 0.0001$ vs. respective baseline using Student t test.

steady-state GIRs correlated inversely with body weight (Supplementary Fig. 1) and visceral adiposity (Fig. 4 and Supplementary Fig. 2).

Effects of Liraglutide, Exercise, and Liraglutide Plus Exercise on Signaling Proteins in Aorta and Muscle

The findings that liraglutide and exercise differentially modulated microvascular insulin responses but synergistically enhanced metabolic insulin actions in HFD-fed rats prompted us to analyze the signaling intermediates involved in insulin action in both the aorta (Supplementary Fig. 3) and skeletal muscle (Supplementary Fig. 4). Exercise or liraglutide alone

significantly enhanced insulin-mediated Akt phosphorylation in aortas, whereas the combination did not. No significant differences were observed in insulin-stimulated eNOS or ERK1/2 phosphorylation in the aorta among the groups. In skeletal muscle, neither insulin-mediated Akt nor ERK phosphorylation differed among the groups. AMPK phosphorylation was comparable among all groups in both the aorta and muscle.

We also examined the protein expression of VEGF and its receptors in the muscle because insulin resistance is associated with decreased capillary density in muscle (34,35), which could affect muscle microvascular perfusion. Neither exercise nor liraglutide, alone or in combination, affected muscle protein

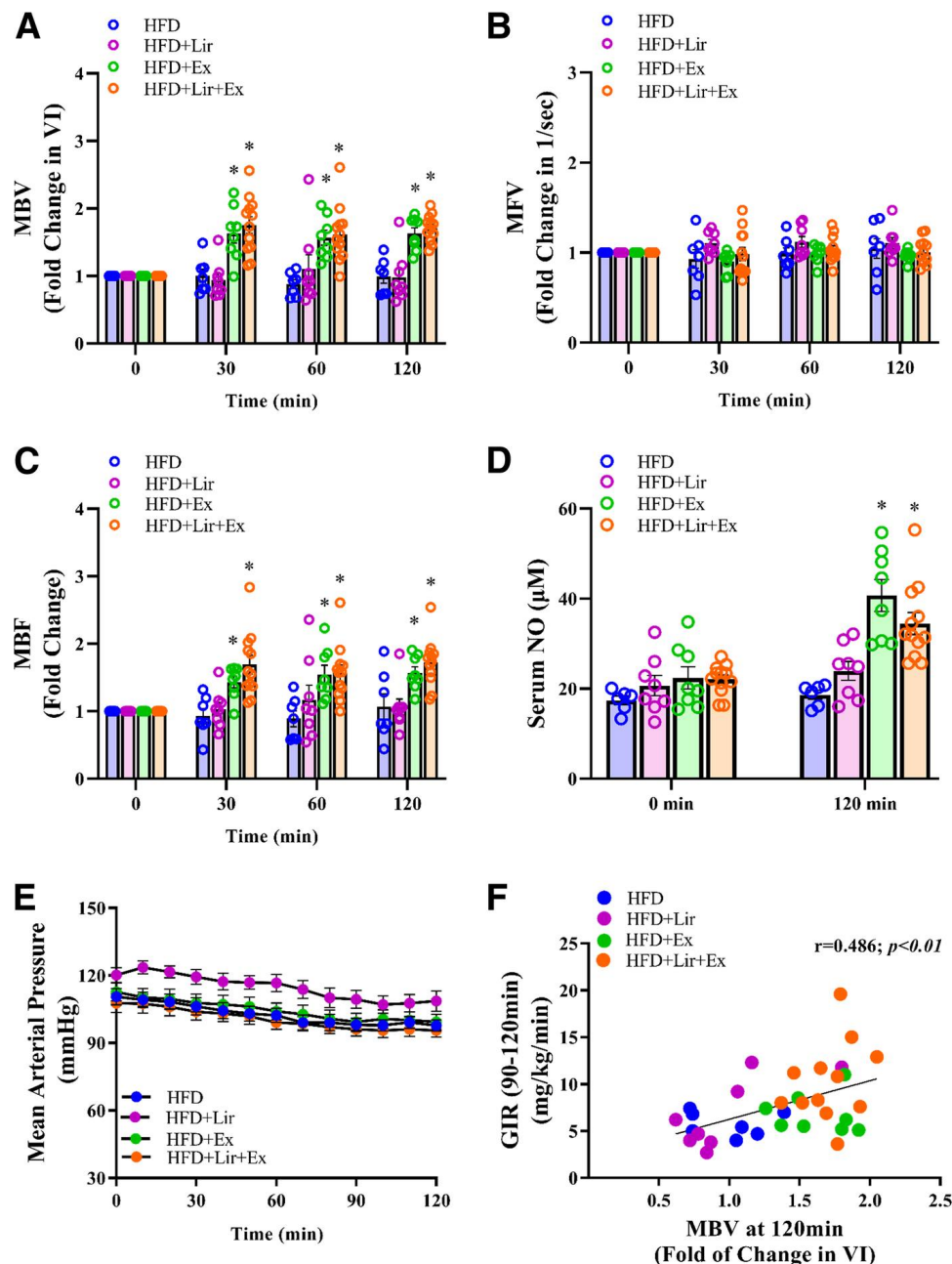


Figure 3—Effects of exercise, with or without liraglutide (Lir), on muscle microvascular insulin responses. A–E: Hindlimb muscle microvascular perfusion was measured using contrast-enhanced ultrasound, with Definity microbubbles as microvascular tracer, before and during insulin clamp (at 0, 30, 60, and 120 min): MBV (A), MFV (B), MBF (C), serum NO levels (D), and Mean arterial blood pressure (E) ($n = 7$ –12 per group). Values are mean $\pm$ SEM. * $P < 0.05$ compared with respective baseline (0 min) using ANOVA with Tukey post hoc analysis. F: Correlation between final MBVs (120 min) and steady-state GIRs by simple linear regression analysis. Ex, exercise; VI, video intensity.

expression of VEGF or its receptors (VEGFR1 and VEGFR2) (Supplementary Fig. 4A and E–G).

Effects of Liraglutide, Exercise, and Liraglutide Plus Exercise on Perimicrovessel Macrophage Accumulation and Superoxide Production in Muscle

Given the critical role of macrophage in the pathogenesis of insulin resistance, we evaluated the effects of liraglutide with or without exercise on muscle CD68⁺ macrophage

accumulation in muscle. Figure 5 shows the number of CD68⁺ cells was comparable in HFD-fed rats with or without liraglutide treatment, but exercise resulted in a significant decrease in CD68⁺ cells ($P < 0.01$). The combined liraglutide and exercise intervention seemed to be more potent in decreasing the accumulation of CD68⁺ cells in muscle, but the difference between combination and exercise alone groups was not statistically significant. Double staining of muscle microvessels with CD31, an indicator of endothelial cells, antibody suggested

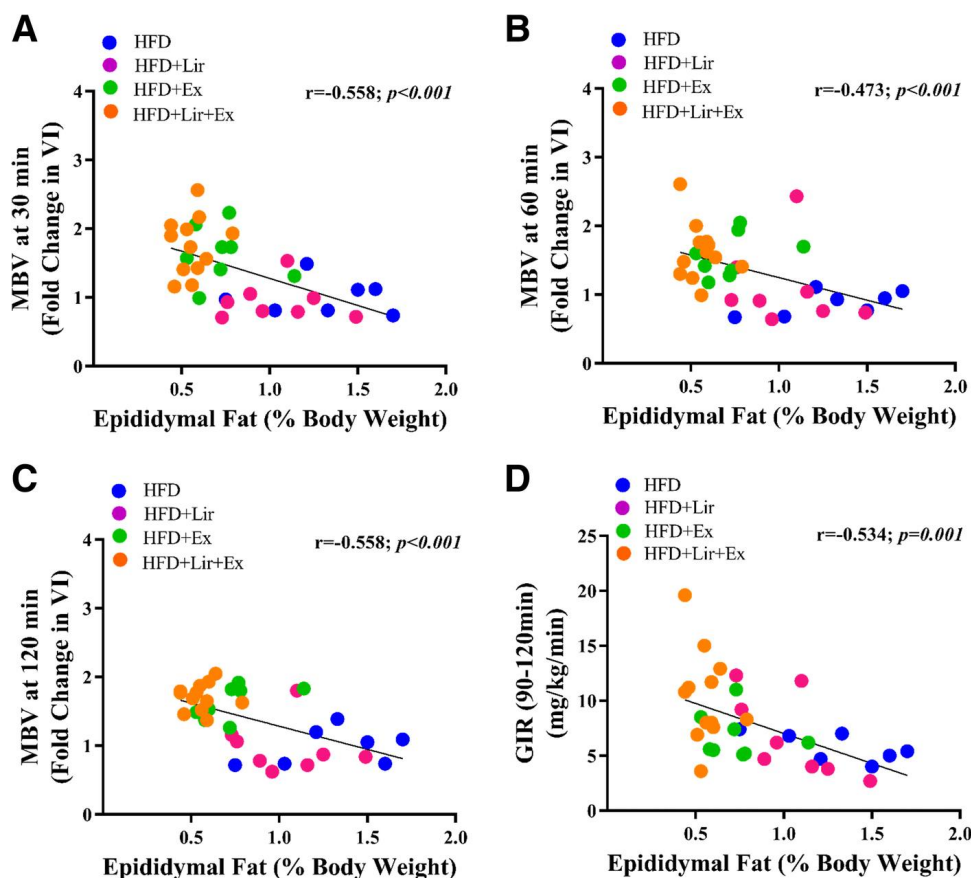


Figure 4—Relationship between visceral adiposity and microvascular or metabolic insulin responses. Bilateral epididymal fat pads in each rat were resected, and weights were normalized to whole body weight. A–D: Correlations between visceral adiposity and MBV at 30 min of insulin clamp (A), visceral adiposity and MBV at 60 min of insulin clamp (B), visceral adiposity and MBV at 120 min of insulin clamp (C), and visceral adiposity and steady-state GIRs (D), all by simple linear regression analysis. Ex, exercise; Lir, liraglutide; VI, video intensity.

that the CD68⁺ cells were predominantly accumulated in the perivascular region. Similarly, exercise and the combined intervention each markedly decreased superoxide levels in muscle, predominantly in the microvessels (Fig. 6). Overall, exercise seems to be the primary driver in the combined intervention group in reducing macrophage accumulation and superoxide production.

Effects of Liraglutide, Exercise, and Liraglutide Plus Exercise on Endothelial Cell Oxidative Stress and AMPK and eNOS Phosphorylation in the Setting of HFD Feeding

We previously demonstrated that inflammation-induced microvascular insulin resistance occurs early in diet-induced obesity (25), and HFD feeding potently induces endothelial cell oxidative stress (29). To examine the impact of various interventions on endothelial oxidative stress, we used immunostaining of CellROX Green Reagent, which is weakly fluorescent in a reduced state and exhibits bright green photostable fluorescence upon oxidation by ROS, to indicate the levels of ROS production and oxidative stress inside the endothelial cells. All three interventions significantly reduced endothelial CellROX Green fluorescence, and exercise, either

alone or in combination with liraglutide, tended to be more effective (Fig. 7A and B). Double staining of endothelial cells with MitoTracker Deep Red, which localizes mitochondria, showed extensive colocalization with CellROX Green staining, suggesting mitochondria were the major source of endothelial ROS. The transcription factor NRF2 translocates to and accumulates in the nucleus upon activation by oxidative stress to induce transcription of target genes that contain the antioxidant response element (36,37). As shown in Fig. 7C and D, the endothelial fluorescence of NRF2 in HFD-fed rats was not affected by liraglutide alone but was significantly enhanced by exercise with or without liraglutide. Colocalization with DAPI staining revealed a markedly increased nuclear accumulation. We also stained the cells for phosphorylated AMPK, which functions as a central regulator of cell survival in response to stressful stimuli, including oxidative stress, and its downstream signaling molecule eNOS (Fig. 7E–H). Liraglutide alone resulted in a significant increase in the phosphorylation of AMPK (Thr¹⁷²) and eNOS (Ser¹¹⁷⁷), whereas exercise alone had a more potent increasing effect. The combined intervention further increased endothelial AMPK, but not eNOS phosphorylation, when compared with exercise alone.

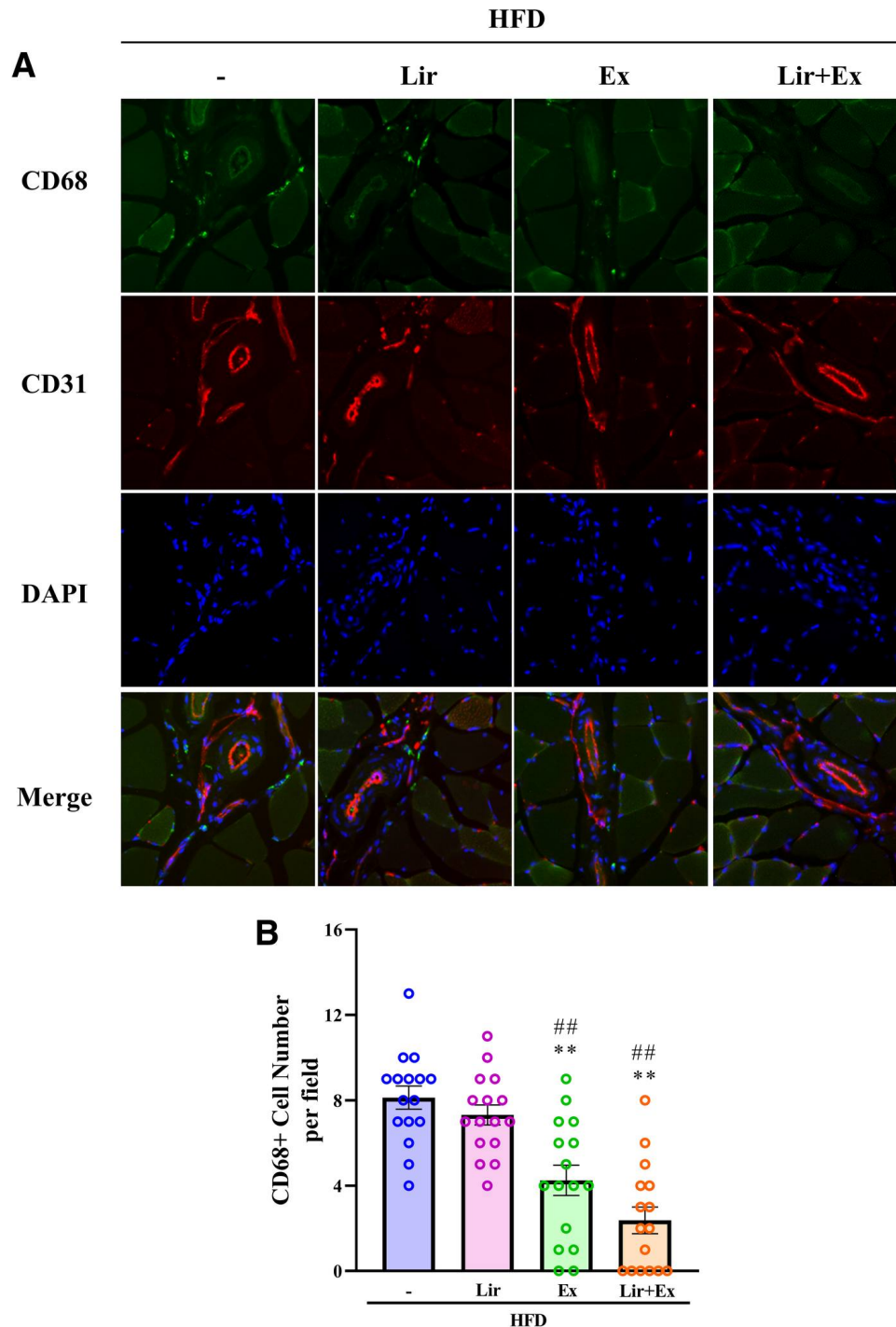


Figure 5—Effects of exercise, with or without liraglutide (Lir), on HFD-induced perivascular macrophage accumulation in the skeletal muscle. *A* and *B*: Representative immunofluorescence staining images (*A*) and quantification (*B*) of CD68⁺ cells. CD31 antibody was used to stain for endothelial cells ($n = 4$ per group). For each sample section, four randomly selected fields were photographed, and CD68⁺ cells were counted and averaged. Values are mean $\pm$ SEM. ** $P < 0.01$ vs. HFD alone group, ## $P < 0.01$ vs. HFD plus Lir group using ANOVA with Tukey post hoc analysis. Ex, exercise.

Effects of Liraglutide, Exercise, and Liraglutide Plus Exercise on Vascular Inflammation and Endothelial Dysfunction

The observation of increased perimicrovessel macrophage accumulation and endothelial cell ROS levels prompted us to examine vascular NF- κ B p65 DNA-binding activity. Liraglutide for 2 weeks did not, whereas exercise slightly decreased

NF- κ B p65 DNA-binding activity in the aorta. However, the liraglutide and exercise combination markedly reduced vascular NF- κ B p65 DNA-binding activity (Fig. 8A). None of the interventions altered NF- κ B p65 DNA-binding activity in muscle. Importantly, this decrease in vascular inflammation was associated with improved endothelial function, assessed using the distal saphenous artery, which is a small resistance

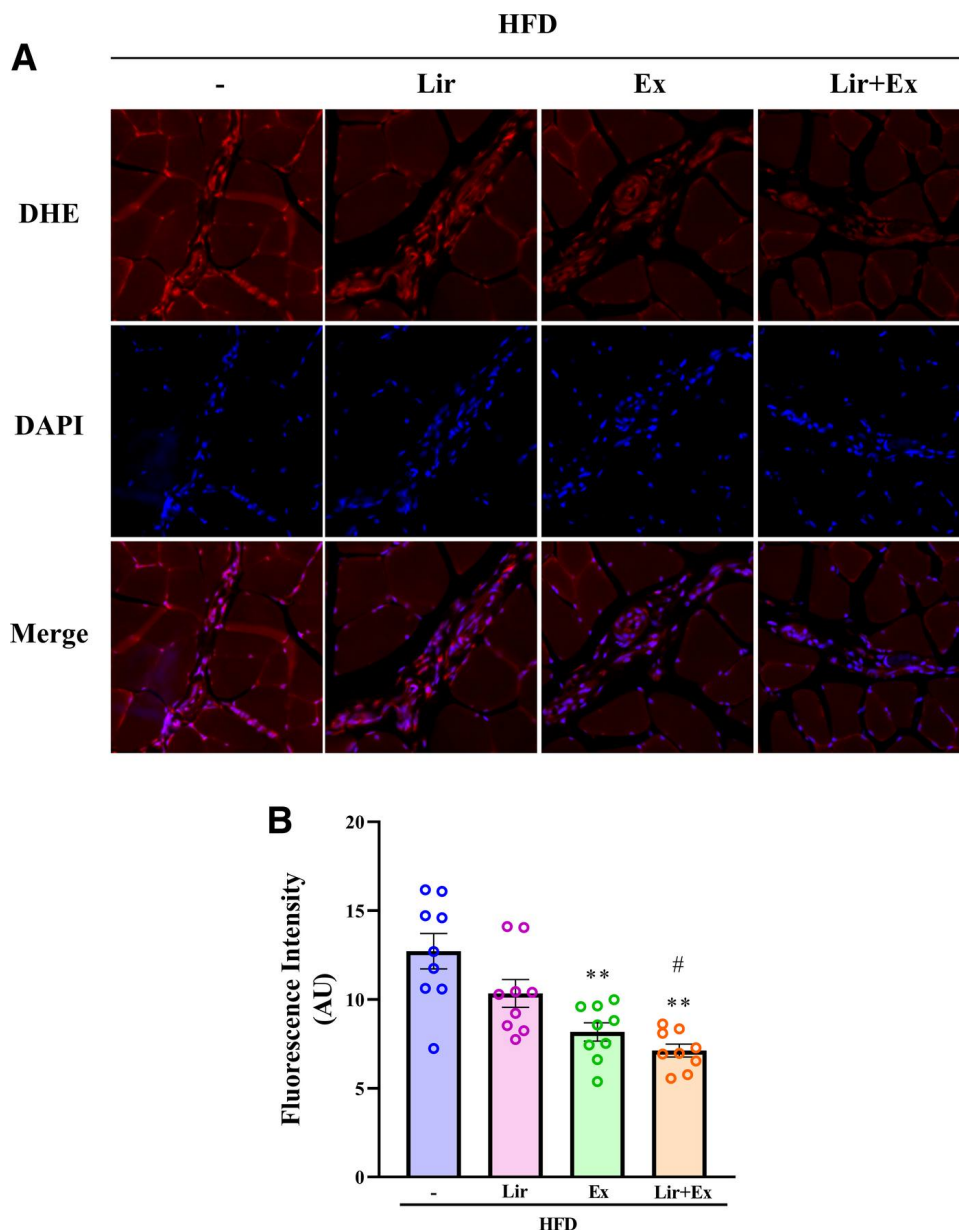


Figure 6—Effects of exercise (Ex), with or without liraglutide (Lir), on HFD-induced superoxide production in the skeletal muscle. **A** and **B**: Representative dihydroethidium (DHE) staining (**A**) and quantitative analysis of the red signal (**B**) in both skeletal muscle and muscle vasculature from HFD-fed rats with various interventions ($n = 3$ per group). For each sample section, three randomly selected fields were photographed, and fluorescence intensity was measured and averaged. Values are mean $\pm$ SEM. $**P < 0.01$ vs. HFD alone group, $\#P < 0.05$ vs. HFD plus Lir group using ANOVA with Tukey post hoc analysis. AU, arbitrary units.

arteriole that feeds the muscle microvasculature (Fig. 8B). Acetylcholine induced significantly more vasodilation at 10^{-5} M in rats receiving exercise interventions with or without liraglutide, and the differences seemed to be primarily driven by exercise. Longer duration of liraglutide treatment, such as for 4 weeks, has been shown to fully rescue endothelial function during HFD (22).

DISCUSSION

The current study demonstrated that, although GLP-1 receptor agonism and exercise each improved metabolic insulin sensitivity, they synergistically enhanced insulin-mediated

glucose disposal in a rodent model of early diet-induced obesity. The combination of exercise and liraglutide intervention was associated with a marked improvement in perimicrovessel macrophage accumulation and superoxide production, endothelial oxidative stress, vascular inflammation, and endothelial dysfunction.

We limited the study intervention to 2 weeks because these animals exhibit significantly higher visceral adiposity without excess weight gain, along with increased fasting plasma insulin concentrations, normal fasting plasma glucose levels, decreased insulin-mediated microvascular recruitment and whole-body glucose disposal, and increased

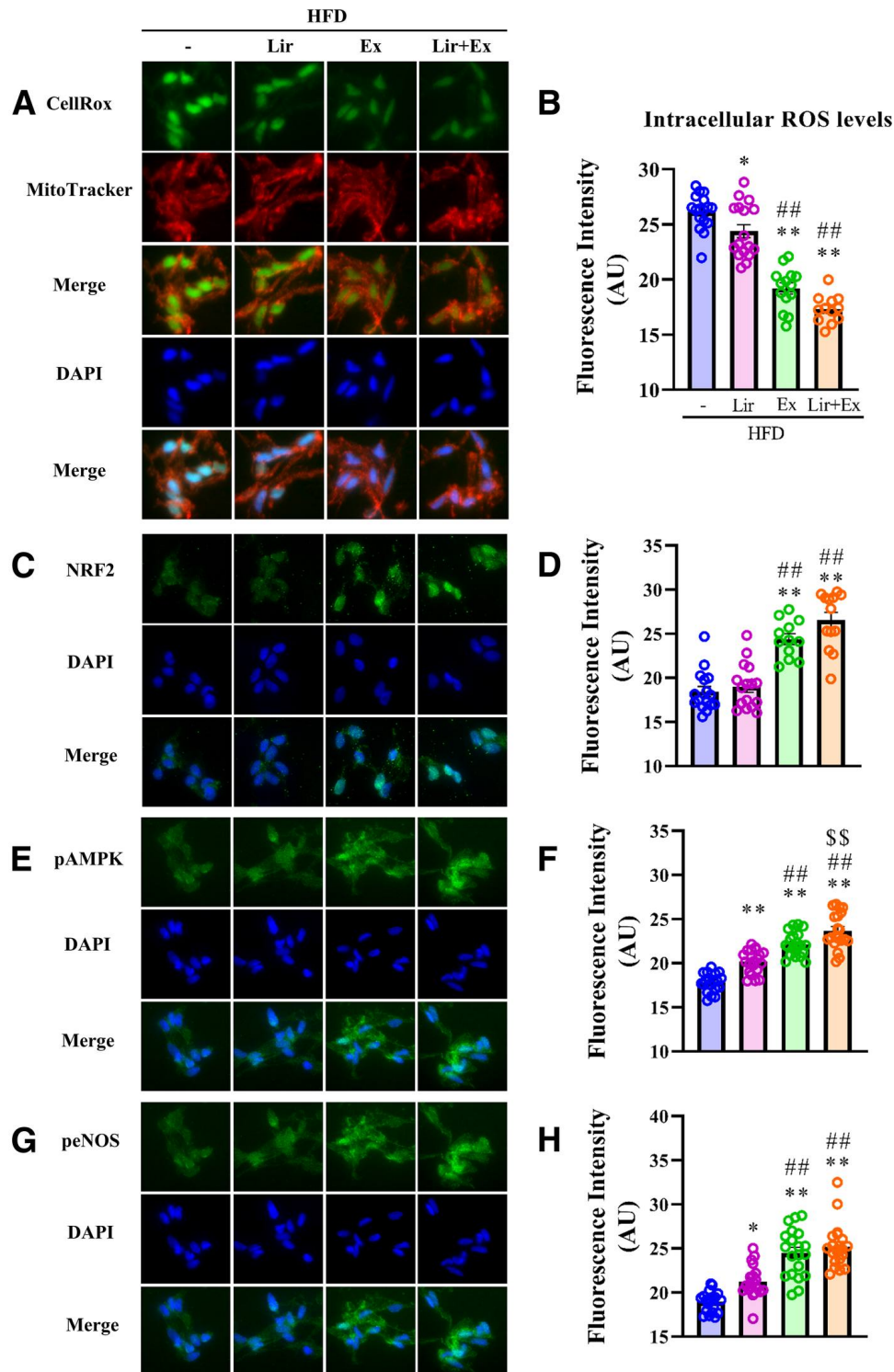


Figure 7—Effects of exercise (Ex), with or without liraglutide (Lir), on endothelial intracellular oxidative stress and AMPK (pAMPK) and eNOS phosphorylation (peNOS). Aortic endothelial cells were freshly isolated from rats at the end of interventions. **A** and **B**: Representative immunofluorescence staining images (**A**) and quantification of intracellular ROS levels (**B**). CellROX Green Reagent was used for ROS-induced oxidative stress detection, and MitoTracker Deep Red was used to stain mitochondria. **C** and **D**: Representative immunofluorescence staining images (**C**) and quantification of NRF2 (**D**). **E** and **F**: Representative immunofluorescence staining images (**E**) and quantification of pAMPK α -1/2 (Thr¹⁷²) (**F**). **G** and **H**: Representative immunofluorescence staining images (**G**) and quantification of peNOS (Ser¹¹⁷⁷) (**H**). For each sample, four to five randomly selected fields were photographed, and fluorescence intensity was measured and averaged ($n = 4$ per group). Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. HFD alone group; ## $P < 0.01$ vs. HFD plus Lir group; \$\$\$ $P < 0.01$ vs. HFD plus Ex group using ANOVA with Tukey post hoc analysis. AU, arbitrary units.

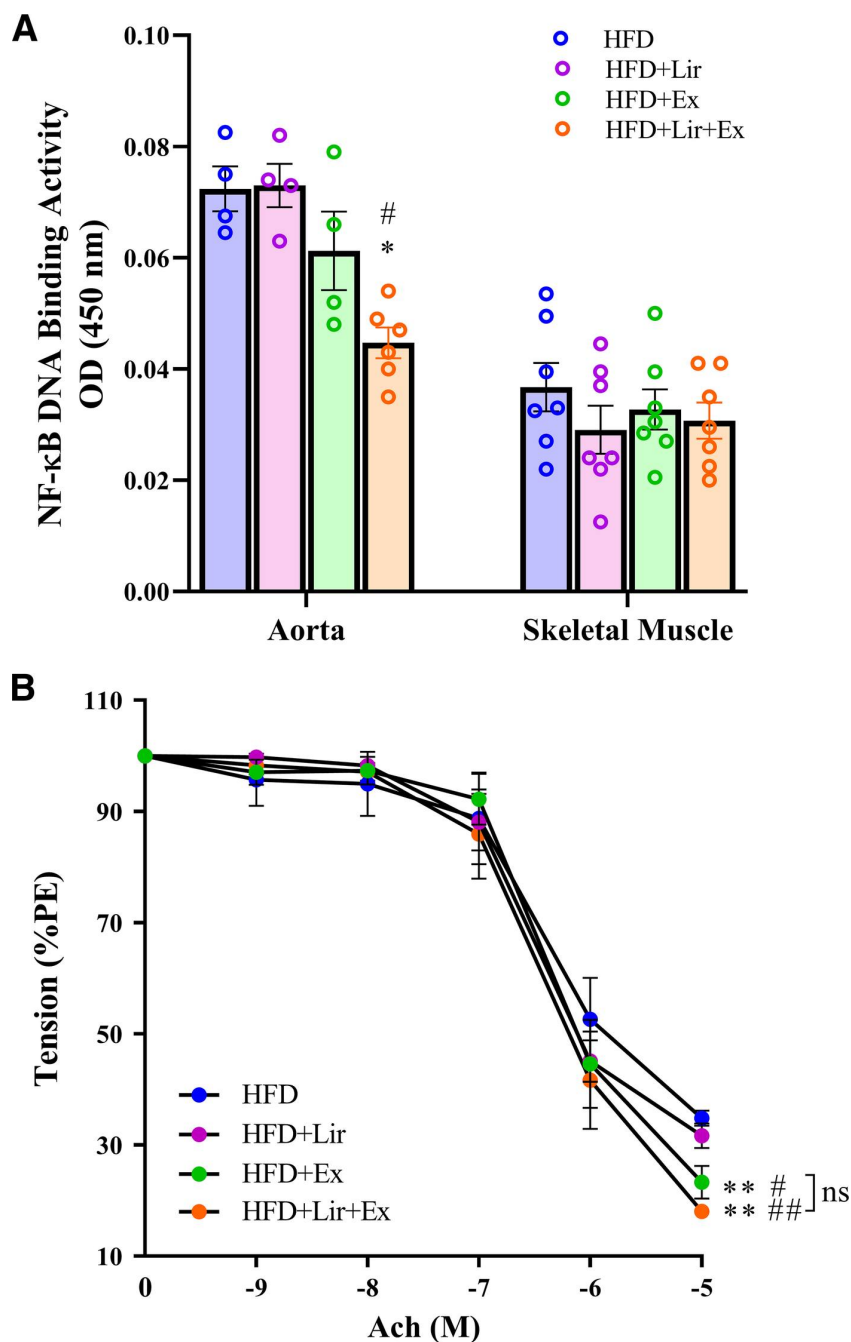


Figure 8—Effects of exercise (Ex), with or without liraglutide (Lir), on HFD-induced endothelial dysfunction and blood vessel NF-κB DNA-binding activity. **A:** Nuclear extracts from aorta ($n = 4$ – 6 per group) and skeletal muscle ($n = 7$ per group) were assayed for NF-κB DNA-binding activity. **B:** Vasodilatory responses to acetylcholine (Ach) were determined using isolated distal saphenous arteries. Each arterial segment was stretched initially to an optimal tension (5 mN) and then allowed to stabilize at baseline tone. After preconstriction with phenylephrine (PE; $2 \mu\text{mol/L}$), vasodilatory responses to Ach were recorded and expressed as percentage of the tension to PE ($n = 5$ – 6 per group). Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. HFD alone group; # $P < 0.05$, ## $P < 0.01$ vs. HFD plus Lir group using ANOVA with Tukey post hoc analysis. OD, optical density.

endothelial ROS content (29). These findings are consistent with the fact that obesity is an inflammatory state and that the vascular endothelium is constantly exposed to circulating inflammatory and insulin resistance-inducing factors, clearly demonstrating the importance of excess body weight in vascular inflammation and oxidative stress and suggesting that preventing excess weight gain might

have contributed to the salutary vascular and metabolic effects observed in the current study. This approach avoids the confounding of excess and prolonged weight gain and allows for a closer examination of the relationship among vascular inflammation, microvascular insulin responses, and metabolic insulin actions in muscle. Rats receiving HFD alone rather than chow-fed rats were used as the

control group because HFD feeding significantly blunts insulin-stimulated microvascular recruitment, whole-body glucose disposal, and aortic Akt and eNOS phosphorylation compared with chow feeding at week 2, and this was associated with a significant increase in aortic NF- κ B activation and endothelial oxidative stress (25,29).

Although lifestyle modification is effective in diabetes prevention, and exercise increases insulin sensitivity (8), animals in the exercise group and combination intervention group consumed a similar amount of food and ran a similar distance during the study duration. Therefore, the amount of food consumption and the exercise level are unlikely to have been the causes of the drastic increase in insulin-mediated glucose disposal seen in the combination intervention group. That the combination intervention markedly decreased body adiposity, reduced serum insulin concentrations, potentially suppressed vascular inflammation and oxidative stress, and improved endothelial function points to a coordinated and synergistic effect that led to the marked increase in insulin-mediated glucose disposal.

Both exercise and GLP-1 receptor agonism are able to reduce oxidative stress, but the mechanisms remain to be defined. Both interventions increase NRF2 activity and activate AMPK, which are two key regulators of redox balance in the cellular cytoprotective response and are abundantly expressed in the endothelium (29,38,39). In the current study, liraglutide and exercise each reduced intracellular ROS levels and increased endothelial AMPK phosphorylation, but exercise seemed to be a stronger effector. Similarly, exercise is more effective in inducing NRF2 nuclear translocation. However, the combination intervention was clearly more effective in reducing endothelial oxidative stress than either intervention alone.

Macrophages contribute to tissue inflammation and play an important role in the pathogenesis of insulin resistance. In the hypothalamus, perivascular macrophages regulate inflammation and mediate vascular homeostasis in response to HFD feeding (40). Perivascular macrophages in muscle are important in the reestablishment of functional tissue perfusion after ischemic injury through the release of inducible NO synthase-produced NO (41). Importantly, inducible NO synthase-mediated NO signaling disrupts lysosomal function and contributes to obesity-associated defective autophagy and insulin resistance in the liver (42). Our observation of the liraglutide and exercise combination potentially reducing perivascular macrophage accumulation in muscle, along with reduced superoxide production, endothelial oxidative stress, and vascular inflammation, argues strongly that perivascular macrophages might be a critical contributor to microvascular inflammation and insulin resistance during obesity development.

In humans at high risk of developing diabetes, liraglutide treatment was associated with 80% less likelihood of developing diabetes (43). In the current study, liraglutide did not alter insulin-mediated microvascular perfusion, endothelial

function, or vascular NF- κ B DNA-binding activity, but it decreased endothelial ROS levels and increased endothelial AMPK and eNOS phosphorylation. That liraglutide treatment reduced body adiposity, which negatively correlated with insulin-mediated MBV, suggests that liraglutide treatment longer than 2 weeks is required to improve microvascular insulin responses. Indeed, simultaneous treatment of rats on an HFD with liraglutide for 4 weeks fully restored microvascular insulin response, improved endothelial function, and attenuated metabolic insulin resistance (22). This is in line with the observation that treatment of HFD-fed *ApoE*^{-/-} mice with liraglutide for 4 weeks improved endothelial function and suppressed vascular smooth muscle cell proliferation by activating AMPK signaling in the aortic wall and inducing cell-cycle arrest (44). With a shorter duration of treatment (2 weeks), our data suggest that exercise is more effective in restoring the microvascular action of insulin and reducing vascular inflammation and oxidative stress and is the main driver in the combination intervention. It is certainly likely that the effect size seen with liraglutide treatment is too small to be detected in the liraglutide alone group and is being masked by the larger effect size of exercise when combined.

Exercise decreased basal insulin concentrations and increased mortality-to-incidence values, suggesting improved insulin sensitivity. That insulin-mediated muscle microvascular perfusion did not further increase, but insulin-stimulated glucose disposal drastically increased, in animals receiving the combination intervention suggests that improvement in insulin-mediated microvascular perfusion only partially contributes to insulin-mediated glucose disposal. Indeed, prior evidence suggests that the microvascular actions of insulin contribute up to 40% of insulin-mediated glucose disposal (6), and expansion of muscle MBV with adiponectin (24,45,46) or GLP-1 (21) or restoration of microvascular insulin action using liraglutide (22) or salicylate (25) improves insulin-mediated glucose disposal by 30–40% in HFD-fed animals. Because insulin resistance occurs at both endothelial and myocytic levels, and endothelial insulin resistance occurs sooner than myocytic insulin resistance (5,15), longer intervention is needed to further restore muscle insulin responses. A previous study showed that HFD feeding takes 8 weeks to increase muscle NF- κ B activity but only 1 week in the aorta in mice (47).

Despite a drastic increase in insulin-mediated glucose disposal in the combination intervention group, insulin-mediated muscle Akt phosphorylation did not differ among all groups. This is not surprising because, although Akt is a key insulin signaling intermediate, there is no clear functional dose-response effect between Akt phosphorylation and muscle insulin action. One study showed in humans with obesity and diabetes reductions of ~60% in insulin-mediated glucose disposal and ~50 and 40% in IRS-1- and IRS-2-associated PI3-kinase activity, respectively, but normal insulin activation of Akt isoforms in muscle (48). In rodents that received lipid infusion, insulin-stimulated Akt

phosphorylation either did not change (31) or changed in an isoform-specific pattern, in that insulin-mediated Akt1 phosphorylation was reduced by 55%, Akt2 phosphorylation paradoxically increased by ~40%, and Akt3 phosphorylation did not change (49). In mice fed an HFD, the basal level of Akt phosphorylation was paradoxically increased, likely from HFD-induced hyperinsulinemia (50).

A limitation of the study is the use of aortic endothelial cells, aortas, and small resistance arterioles instead of entirely microvessels and microvascular endothelial cells. However, the current approach allowed us to examine the impact of various interventions on conduit as well as resistance vessels, given the well-known effects of HFD on vascular inflammation and function in these vessels, and changes in conduit and resistance vessel functions subsequently affect microvascular perfusion. Additionally, it is very difficult to isolate a sufficient amount of endothelial cells from skeletal muscle for the proposed signaling studies. Also, endothelial cells across the arterial tree seem to respond to inflammatory and insulin resistance-inducing factors similarly in the literature, likely because of the unique geographical location (lining the vessel) and a specific barrier function between the circulatory compartment and tissue interstitium, and as such, all endothelial cells irrespective of location are constantly exposed to circulating factors.

In conclusion, both liraglutide alone and exercise alone increased muscle insulin sensitivity during obesity development, but the combination of both synergistically and drastically improved metabolic insulin sensitivity, along with markedly reducing perivascular macrophage accumulation and superoxide production in muscle, endothelial oxidative stress, and vessel inflammation. Our data argue strongly that early combinational intervention with exercise and GLP-1 receptor agonism could effectively prevent insulin resistance and associated metabolic disarrays.

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References

1. Liu Z. The vascular endothelium in diabetes and its potential as a therapeutic target. *Rev Endocr Metab Disord* 2013;14:1–3
2. Barrett EJ, Eggleston EM, Inyard AC, et al. The vascular actions of insulin control its delivery to muscle and regulate the rate-limiting step in skeletal muscle insulin action. *Diabetologia* 2009;52:752–764

3. Barrett EJ, Liu Z. The endothelial cell: an “early responder” in the development of insulin resistance. *Rev Endocr Metab Disord* 2013;14:21–27
4. Barrett EJ, Wang H, Upchurch CT, Liu Z. Insulin regulates its own delivery to skeletal muscle by feed-forward actions on the vasculature. *Am J Physiol Endocrinol Metab* 2011;301:E252–E263
5. Love KM, Barrett EJ, Malin SK, Reusch JEB, Regensteiner JG, Liu Z. Diabetes pathogenesis and management: the endothelium comes of age. *J Mol Cell Biol* 2021;13:500–512
6. 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* 2003;285:E123–E129
7. DeFronzo RA, Gunnarsson R, Björkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J Clin Invest* 1985;76:149–155
8. Zheng C, Liu Z. Vascular function, insulin action, and exercise: an intricate interplay. *Trends Endocrinol Metab* 2015;26:297–304
9. Scognamiglio R, Negut C, de Kreutzenberg SV, Palisi M, Tiengo A, Avogaro A. Abnormal myocardial perfusion and contractile recruitment during exercise in type 1 diabetic patients. *Clin Cardiol* 2005;28:93–99
10. Vincent MA, Clerk LH, Lindner JR, et al. Mixed meal and light exercise each recruit muscle capillaries in healthy humans. *Am J Physiol Endocrinol Metab* 2006;290:E1191–E1197
11. Inyard AC, Clerk LH, Vincent MA, Barrett EJ. Contraction stimulates nitric oxide independent microvascular recruitment and increases muscle insulin uptake. *Diabetes* 2007;56:2194–2200
12. Hoier B, Hellsten Y. Exercise-induced capillary growth in human skeletal muscle and the dynamics of VEGF. *Microcirculation* 2014;21:301–314
13. Wheatley CM, Rattigan S, Richards SM, Barrett EJ, Clark MG. Skeletal muscle contraction stimulates capillary recruitment and glucose uptake in insulin-resistant obese Zucker rats. *Am J Physiol Endocrinol Metab* 2004;287:E804–E809
14. Nyström T, Gutniak MK, Zhang Q, et al. Effects of glucagon-like peptide-1 on endothelial function in type 2 diabetes patients with stable coronary artery disease. *Am J Physiol Endocrinol Metab* 2004;287:E1209–E1215
15. Love KM, Liu J, Regensteiner JG, Reusch JEB, Liu Z. GLP-1 and insulin regulation of skeletal and cardiac muscle microvascular perfusion in type 2 diabetes. *J Diabetes* 2020;12:488–498
16. Chai W, Dong Z, Wang N, et al. Glucagon-like peptide 1 recruits microvasculature and increases glucose use in muscle via a nitric oxide-dependent mechanism. *Diabetes* 2012;61:888–896
17. Subaran SC, Sauder MA, Chai W, et al. GLP-1 at physiological concentrations recruits skeletal and cardiac muscle microvasculature in healthy humans. *Clin Sci (Lond)* 2014;127:163–170
18. Tan AWK, Subaran SC, Sauder MA, et al. GLP-1 and insulin recruit muscle microvasculature and dilate conduit artery individually but not additively in healthy humans. *J Endocr Soc* 2018;2:190–206
19. Dong Z, Chai W, Wang W, et al. Protein kinase A mediates glucagon-like peptide 1-induced nitric oxide production and muscle microvascular recruitment. *Am J Physiol Endocrinol Metab* 2013;304:E222–E228
20. Wang N, Tan AWK, Jahn LA, et al. Vasodilatory actions of glucagon-like peptide 1 are preserved in skeletal and cardiac muscle microvasculature but not in conduit artery in obese humans with vascular insulin resistance. *Diabetes Care* 2020;43:634–642
21. Chai W, Zhang X, Barrett EJ, Liu Z. Glucagon-like peptide 1 recruits muscle microvasculature and improves insulin's metabolic action in the presence of insulin resistance. *Diabetes* 2014;63:2788–2799
22. Chai W, Fu Z, Aylor KW, Barrett EJ, Liu Z. Liraglutide prevents microvascular insulin resistance and preserves muscle capillary density in high-fat diet-fed rats. *Am J Physiol Endocrinol Metab* 2016;311:E640–E648
23. Chai W, Wang W, Liu J, et al. Angiotensin II type 1 and type 2 receptors regulate basal skeletal muscle microvascular volume and glucose use. *Hypertension* 2010;55:523–530

24. Zhao L, Fu Z, Wu J, et al. Globular adiponectin ameliorates metabolic insulin resistance via AMPK-mediated restoration of microvascular insulin responses. *J Physiol* 2015;593:4067–4079
25. Zhao L, Fu Z, Wu J, et al. Inflammation-induced microvascular insulin resistance is an early event in diet-induced obesity. *Clin Sci (Lond)* 2015;129:1025–1036
26. Fu Z, Zhao L, Chai W, Dong Z, Cao W, Liu Z. Ranolazine recruits muscle microvasculature and enhances insulin action in rats. *J Physiol* 2013;591:5235–5249
27. Fu Z, Zhao L, Aylor KW, Carey RM, Barrett EJ, Liu Z. Angiotensin-(1-7) recruits muscle microvasculature and enhances insulin's metabolic action via mas receptor. *Hypertension* 2014;63:1219–1227
28. US Department of Health, Education and Welfare. Guide for the Care and Use of Laboratory Animals. Bethesda, MD, National Institutes of Health, 1996 (publ. no. 85-23)
29. Liu J, Aylor KW, Chai W, Barrett EJ, Liu Z. Metformin prevents endothelial oxidative stress and microvascular insulin resistance during obesity development in male rats. *Am J Physiol Endocrinol Metab* 2022;322:E293–E306
30. Zhang W-J, Wei H, Hagen T, Frei B. Alpha-lipoic acid attenuates LPS-induced inflammatory responses by activating the phosphoinositide 3-kinase/Akt signaling pathway. *Proc Natl Acad Sci USA* 2007;104:4077–4082
31. Wang N, Chai W, Zhao L, Tao L, Cao W, Liu Z. Losartan increases muscle insulin delivery and rescues insulin's metabolic action during lipid infusion via microvascular recruitment. *Am J Physiol Endocrinol Metab* 2013;304:E538–E545
32. Liu J, Liu Z. Muscle insulin resistance and the inflamed microvasculature: fire from within. *Int J Mol Sci* 2019;20:562
33. Love KM, Jahn LA, Hartline LM, Patrie JT, Barrett EJ, Liu Z. Insulin-mediated muscle microvascular perfusion and its phenotypic predictors in humans. *Sci Rep* 2021;11:11433
34. Lillioja S, Young AA, Culter CL, et al. Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *J Clin Invest* 1987;80:415–424
35. Chung AWY, Hsiang YN, Matzke LA, McManus BM, van Breemen C, Okon EB. Reduced expression of vascular endothelial growth factor paralleled with the increased angiostatin expression resulting from the upregulated activities of matrix metalloproteinase-2 and -9 in human type 2 diabetic arterial vasculature. *Circ Res* 2006;99:140–148
36. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 2007;47:89–116
37. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc Natl Acad Sci USA* 1996;93:14960–14965
38. Dai G, Vaughn S, Zhang Y, Wang ET, Garcia-Cardena G, Gimbrone MA Jr. Biomechanical forces in atherosclerosis-resistant vascular regions regulate endothelial redox balance via phosphoinositol 3-kinase/Akt-dependent activation of Nrf2. *Circ Res* 2007;101:723–733
39. Fledderus JO, Boon RA, Volger OL, et al. KLF2 primes the antioxidant transcription factor Nrf2 for activation in endothelial cells. *Arterioscler Thromb Vasc Biol* 2008;28:1339–1346
40. Mendes NF, Velloso LA. Perivascular macrophages in high-fat diet-induced hypothalamic inflammation. *J Neuroinflammation* 2022;19:136
41. Vågesjö E, Parv K, Ahl D, et al. Perivascular macrophages regulate blood flow following tissue damage. *Circ Res* 2021;128:1694–1707
42. Qian Q, Zhang Z, Li M, et al. Hepatic lysosomal iNOS activity impairs autophagy in obesity. *Cell Mol Gastroenterol Hepatol* 2019;8:95–110
43. le Roux CW, Astrup A, Fujioka K, et al. SCALE Obesity Prediabetes NN8022-1839 Study Group. 3 years of liraglutide versus placebo for type 2 diabetes risk reduction and weight management in individuals with prediabetes: a randomised, double-blind trial. *Lancet* 2017;389:1399–1409
44. Jojima T, Uchida K, Akimoto K, et al. Liraglutide, a GLP-1 receptor agonist, inhibits vascular smooth muscle cell proliferation by enhancing AMP-activated protein kinase and cell cycle regulation, and delays atherosclerosis in ApoE deficient mice. *Atherosclerosis* 2017;261:44–51
45. Zhao L, Chai W, Fu Z, et al. Globular adiponectin enhances muscle insulin action via microvascular recruitment and increased insulin delivery. *Circ Res* 2013;112:1263–1271
46. Zhao L, Fu Z, Liu Z. Adiponectin and insulin cross talk: the microvascular connection. *Trends Cardiovasc Med* 2014;24:319–324
47. Kim F, Pham M, Maloney E, et al. Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance. *Arterioscler Thromb Vasc Biol* 2008;28:1982–1988
48. Kim Y-B, Nikoulina SE, Ciaraldi TP, Henry RR, Kahn BB. Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J Clin Invest* 1999;104:733–741
49. Kim Y-B, Shulman GI, Kahn BB. Fatty acid infusion selectively impairs insulin action on Akt1 and protein kinase C lambda /zeta but not on glycogen synthase kinase-3. *J Biol Chem* 2002;277:32915–32922
50. Liu H-Y, Hong T, Wen G-B, et al. Increased basal level of Akt-dependent insulin signaling may be responsible for the development of insulin resistance. *Am J Physiol Endocrinol Metab* 2009;297:E898–E906