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Effect of Limb Demand Ischemia on Autophagy and Morphology in Mice

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Abstract

Background—Obesity is a major risk factor for diabetes and peripheral arterial disease (PAD), which frequently leads to lower limb demand ischemia. Skeletal muscle autophagy and mitochondrial biogenesis are important processes for proper oxidative capacity and energy metabolism which are compromised in diabetes. This study compares autophagy, mitochondrial biogenesis, energy metabolism, and morphology in the hind limbs of obese diabetic mice subjected to demand or sedentary ischemia.

Materials and Methods—Unilateral hind limb demand ischemia was created in a group of diet induced obese mice following femoral artery ligation (FAL) and 4 weeks of daily exercise. A parallel group of mice underwent FAL but remained sedentary for 4 weeks. Hind limbs muscles were analyzed for markers of autophagy, mitochondrial biogenesis, ATP, and muscle tissue morphology.

Results—At the end of the 4-week exercise period, demand ischemia increased the autophagy mediator Beclin-1, but it did not alter the autophagy indicator LC3B-II/I ratio, or markers of

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mitochondrial biogenesis, Opa-1/Drp-1. In contrast, exercise significantly increased the level of mitochondrial protein-SDHA and reduced adipocyte accumulation and the percentage of centrally nucleated myofibers in the demand ischemia limb. In addition, demand ischemia resulted in decreased uncoupling protein-3 levels without altering muscle ATP or pS473-Akt levels.

Conclusions—Limb demand ischemia markedly decreased adipocyte accumulation and enhanced muscle regeneration in obese mice but it did not appear to enhance autophagy, mitochondrial biogenesis, energy metabolism, or insulin sensitivity. Future studies aimed at evaluating novel therapies that enhance autophagy and mitochondrial biogenesis in diabetes with PAD are warranted.

Keywords

limb demand ischemia; diet induced obesity; Type-2 Diabetes; peripheral arterial disease; autophagy; mitochondrial biogenesis; muscle regeneration

Introduction

Obesity and the metabolic syndrome predispose patients to the development of diabetes and peripheral arterial disease (PAD). In obese patients, demand ischemia-induced claudication worsens their prognosis, independent from other compounding factors^{1, 2}. Pathologically, demand ischemia in patients with PAD causes repetitive cycles of ischemic injury that exacerbate mitochondrial dysfunction, which induces oxidative stress³ and gait deficits⁴. Similar to muscle specimens from PAD patients, mice with chronic hind limb ischemia display evidence of myopathy associated with mitochondrial dysfunction and oxidative damage⁵. A correlation has been established between the severity of PAD, diminished lower limb skeletal muscle protein levels, and mitochondrial content in the diseased limb⁶. Histological studies characterizing the morphology of skeletal muscle specimens of PAD patients^{7, 8} have revealed evidence of myofiber degeneration with variable levels of fibrosis and fatty deposition⁹. The etiology of type-2 diabetes, a major risk factor for PAD, has been linked with skeletal muscle insulin resistance, but the exact mechanism requires further investigation. Substantial evidence has implicated increased oxidative stress, mitochondrial dysfunction, and intramuscular fat accumulation as the primary targets¹⁰. Recently, research has focused on investigating the role of autophagy and defective mitochondrial biogenesis in the pathogenesis of insulin resistance in ageing¹¹, and in the metabolic syndrome with the associated comorbidities in cardiovascular disease^{12, 13}. Autophagy and mitochondrial biogenesis are synchronized processes important in the regulation of energy metabolism and cellular homeostasis that also provide tissue protective mechanism under stress or pathologic conditions¹⁴. Consisting of several cellular degradation pathways, autophagy targets partially degraded or aged cytoplasmic materials or organelles including mitochondria for lysosomal enzymatic digestion and recycling. Mitochondrial biogenesis consists of mitochondrial fusion and fission which are particularly important in bioenergetics, tissue remodeling and function of skeletal muscle¹⁵.

To date, limited literature is available on the effects of exercise on limb muscle autophagy and morphology after demand ischemia in the setting of type-2 diabetes. We hypothesized that the pattern of molecular responses associated with autophagy, mitochondrial biogenesis,

energy metabolism, and muscle morphology would be different in demand ischemia compared to sedentary ischemia.

Therefore, the purpose of this study was to evaluate the expression of selected regulatory proteins involved in autophagy and mitochondrial dynamics in diet induced obese diabetic mice subjected to demand or sedentary ischemia after a 4-week exercise or sedentary period. This study also aimed to elucidate the effect of a prolonged period of daily exercise on energy substrate levels, insulin sensitization, morphologic indices of skeletal muscle regeneration, including pathologic adipocyte accumulation.

Materials and Methods

Animal Protocol

Animal care and experimental procedures were in compliance with the “Principal of Laboratory Animal Care” (Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication 86-23, 1985) and approved by the Institutional Review Committee. Age-matched C57BL6 male mice were acquired from Jackson Laboratory (Bar Harbor, ME) after being fed a diet consisting of 60% kcal from fat for 26 weeks to induce obesity, insulin resistance, and diabetes¹⁶. To induce hind limb ischemia, all mice were subjected to unilateral femoral artery ligation surgery (FAL) in one hind limb or sham surgery in the contralateral hind limb and then were allowed to recover for two weeks as previously described^{17, 18}. Subsequently, the mice were divided into two groups; exercise group and sedentary group. The exercise group (n=8) underwent 60 minutes of daily treadmill exercise (12 meters/minute speed at 10° incline) for 4 weeks to induce demand ischemia condition in the hind limb that underwent FAL and exercised non-ischemic contralateral hind limb in each mouse. The sedentary group (n=7) were not exercised for 4 weeks after the recovery period following FAL thus creating the sedentary ischemia in the hind limb that had FAL and sedentary non-ischemic contralateral hind limb in each animal. The activity of the sedentary mice was not restricted in the cage. Whole body weight and hind limb laser Doppler imaging was documented at baseline before surgery and once a week thereafter. Hind limb muscle tissues were harvested at the end of the 4-week exercise or sedentary period. Collected tissues were divided then either fixed in paraformaldehyde for histological evaluation or stored at -80°C for molecular analyses.

SDS-PAGE immunoblotting for markers of autophagy, mitochondrial biogenesis, phosphorylated-Akt, and UCP-3

100µg soluble protein aliquots isolated from hind limb tissue using RIPA extraction buffer were subjected to Tris-HCl-SDS electrophoresis and electroblotting transfer followed by chemiluminescence detection as previously described¹⁶. To detect protein markers of autophagy, the membranes were incubated with an IgG against the microtubule-associated protein light chain-3B (LC3B, Cell Signaling, Danvers, MA), and rabbit monoclonal anti-Beclin-1 (Cell Signaling, Danvers, MA). To assess markers of mitochondrial biogenesis, membranes were incubated with rabbit polyclonal anti-Optic Atrophy-1 (Opa-1) IgG (Millipore, Billerica, MA) and rabbit monoclonal anti-Dynamin-Related Protein-1 (Drp-1) IgG (Cell Signaling, #5391, Danvers, MA). To evaluate the mitochondrial protein content,

membranes were probed with mouse antibody that recognizes the mitochondrial complex II protein: succinate dehydrogenase subunit-A (SDHA, AbCam, Cambridge, MA) or rabbit polyclonal anti-cytochrome-c IgG (Cell Signaling, Danvers, MA). To evaluate PI3/Akt pathway activity we assessed the level of phosphorylated-Akt, using rabbit monoclonal IgG that recognize pS473-Akt (Cell Signaling, Danvers, MA). The level of the mitochondrial uncoupling protein-3 (UCP-3), was measured by probing the membranes with a rabbit anti-UCP-3 IgG (Thermo-Fisher Scientific, Waltham, MA). The specific band integrated density values for each membrane were measured using FluorChem HD2 imaging software, which normalized the values to the bands densities acquired from Ponceau-S staining (Sigma-Aldrich, St Louis, MO) and results are expressed as arbitrary unit (AU) as previously described¹⁶.

Morphometric analysis of hind limb skeletal muscle

To determine the changes in muscle fiber morphology, tissues were dehydrated in graded acetone and imbedded in JB-4 polymer (Polysciences Inc, Warrington, PA). 2µm thick cross sections were obtained from the middle segment of the tibialis anterior (TA) muscle. The muscle sections were stained with H&E and randomized digital microscope images were obtained at 200× magnification and examined by a blinded observer. The fat cell area was measured and the percentage of fibers presenting a central nucleus was determined as an indication of immature myofibers as previously described^{16, 19, 20}.

Muscle adenosine triphosphate (ATP) quantification

ATP levels in skeletal muscle tissue were measured after homogenization in 10% trichloroacetic acid using the ATPlite luminescence assay (PerkinElmer Life, Waltham, MA) following the manufacturer's instructions as previously described¹⁶. ATP levels were expressed as nanomoles per milligram of total protein.

Immunohistochemistry detection of UCP-3 in skeletal muscle tissues

To detect the uncoupling protein-3, hind limb muscle tissues were harvested and fixed in formalin for 24 hours followed by graded alcohol dehydration and paraffin imbedding. 8 µm cross sections were generated and incubated with 1:500 dilution of rabbit anti-UCP-3 polyclonal IgG (Thermo-Fisher Scientific, Waltham, MA) followed by incubation with goat anti rabbit-horseradish peroxidase conjugated IgG at 1:300 dilution and UCP-3 protein visualization with 3,3'-Diaminobenzidine substrate (DAB solution kit, Vector Laboratories, Burlingame, CA). The tissue sections were counterstained with Hematoxylin and digital images were acquired at 200× magnification.

Statistical analysis

Statistical analysis was performed with Graph pad Prism-5 (Graph Pad, San Diego, CA) using parametric or non-parametric unpaired t-test or ANOVA. The results were expressed as the average ± standard error of the mean.

Results

Assessment of hind limbs perfusion and change in body weight after exercise

The perfusion index (flux ratio) was assessed in the hind limbs at baseline, immediately after FAL or sham surgery, and once a week thereafter until the end of the 4-week exercise or sedentary period (Figure-1A). Both groups demonstrated a remarkable reduction in hind limb perfusion immediately after FAL surgery. Ischemic hind limb perfusion gradually recovered over the following weeks but remained significantly lower by ~50% compared to the initial baseline levels ($p < 0.001$, Figure-1A). The hind limb perfusion ratio did not differ between the exercised and the sedentary groups at any time point. Change in whole body weight in both groups of mice was determined between the baseline weight of animals before the initiation of the exercised/sedentary state and the weight at the end of 4-week experimental period. Mice that were exercised and experienced demand ischemia exhibited substantially less weight gain over the course of the 4-week experimental period when compared to the sedentary mice ($*p < 0.0001$, Figure-1B). These data indicate that exercise significantly reduced weight gain but did not alter hind limb perfusion in the diabetic obese mice fed a high fat diet.

Effect of demand ischemia and exercise on indices of hind limb muscle autophagy

The microtubule-associated protein LC3, the first identified mammalian protein localized in the autophagosome membrane is widely used as a marker for autophagosome formation. LC3 protein is present in two forms: the cystolic form, LC3-I, and the lipidated form, LC3-II, which is located on the autophagosomal membrane. The ratio of LC3-II to LC3-I indicates the degree of autophagosome formation. Our results show that the LC3-II/LC3-I ratio was significantly increased in the exercised non-ischemic contralateral muscle compared to sedentary nonischemic contralateral muscle indicating a higher autophagy rate (LC3B II/I; Sedentary Contralateral: 0.27 ± 0.04 vs. Exercised Contralateral: 0.51 ± 0.072 AU, $p < 0.05$, Figure-2B). In contrast, a trend of higher LC3-II/LC3-I ratio in demand ischemia compared to sedentary ischemia was observed but it did not reach statistical significance (LC3B II/I: Sedentary Ischemia; 0.22 ± 0.019 AU, vs. Demand Ischemia; 0.39 ± 0.069 AU, ns, Figure-2B). These data suggest that exercise upregulated autophagy in the non-ischemic muscle, but it failed to significantly enhance autophagy in the limb subjected to demand ischemia. Beclin-1, is an important autophagy mediator, has been identified in mammalian species. The results indicated a significant increase in Beclin-1 protein levels in both the exercised non-ischemic contralateral muscle and demand ischemia muscle when compared to sedentary non-ischemic contralateral muscles (Beclin-1: Sedentary Contralateral; 1.707 ± 0.06 vs. Exercised Contralateral; 2.81 ± 0.25 AU, $p < 0.01$ and vs. Demand Ischemia; 3.04 ± 0.29 AU, $p < 0.01$, Figure-2C).

Effect of demand ischemia and exercise on mitochondrial biogenesis regulatory proteins

Mitochondrial fusion of the outer and inner mitochondrial membranes is mediated by GTPase proteins: mitofusin-1, mitofusin-2, and Opa-1. Conversely, mitochondrial fission requires that proteins, Drp-1 and Fis-1, assemble at fission sites on the mitochondrial membrane to induce membrane division¹². Our data showed that the expression ratio of the mitochondrial fusion, Opa-1, to the mitochondrial fission protein, Drp-1, was not different

between the groups. These data suggest that exercise did not alter the balance of mitochondrial fission/fusion proteins between the groups in these mice (Opa-1/Drp-1; Sedentary Contralateral: 1.96 ± 0.59 vs. Exercised Contralateral: 2.03 ± 0.35 AU, and Sedentary Ischemia: 2.36 ± 0.95 vs. Demand Ischemia: 1.44 ± 0.14 AU, Figure-2D).

Effect of demand ischemia and exercise on mitochondrial proteins contents

The protein level of the mitochondrial complex-II associated protein, SDHA was significantly increased in the exercised contralateral and demand ischemia muscle compared to sedentary contralateral muscle (SDHA: Sedentary Contralateral, 0.47 ± 0.03 vs. Exercised Contralateral: 0.57 ± 0.03 AU, $p < 0.05$, and vs. Demand Ischemia: 0.58 ± 0.02 AU, $p < 0.01$, Figure-3B). In contrast, there was no difference in SDHA between the sedentary ischemia and sedentary contralateral muscle (SDHA; Sedentary Contralateral, 0.47 ± 0.03 vs. Sedentary Ischemia, 0.43 ± 0.01 AU, Figure-3B). In addition, Cytochrome-C levels were significantly enhanced in the exercised contralateral muscles compared to sedentary contralateral and demand ischemia (Exercised Contralateral; 0.65 ± 0.09 vs. Sedentary Contralateral; 0.41 ± 0.06 AU, $p < 0.05$, and Demand Ischemia; 0.38 ± 0.03 AU, $p < 0.05$, Figure-3C). There was no difference in the Cytochrome-C levels between the demand ischemia and sedentary ischemia muscles (Demand Ischemia; 0.38 ± 0.03 vs. Sedentary Ischemia; 0.34 ± 0.08 AU, ns, Figure-3C). This data suggests that exercise enhances the level of mitochondrial proteins in the non-ischemic exercised muscle but alters selected proteins in the ischemic muscle.

Effect of demand ischemia and exercise on muscle fiber morphology

Examination of the stained TA muscle cross sections from the hind limbs of sedentary ischemic mice showed extensive adipocyte accumulation and myofibers that presented a central nucleus indicating delayed fiber maturation (Figure-4A). However, demand ischemia muscle, exhibited a marked reduction of both adipocyte accumulation and centrally nucleated myofibers (Figure-4B). Quantitative analysis of the average adipocyte area per high power field demonstrated significantly lower average area in the muscles subjected to demand ischemia compared to sedentary ischemia (Sedentary Ischemia: 21356.7 ± 6739.5 vs. Demand Ischemia: $5181.1 \pm 852.8 \mu\text{m}^2$, $p = 0.015$, Figure-4C). In addition, demand ischemia exhibited a significant reduction in the percentage of centrally nucleated myofibers per field when compared to sedentary ischemia muscle (Sedentary Ischemia: 59.6 ± 2.9 vs. Demand Ischemia: 37.9 ± 5.8 percent central nuclei, $p = 0.01$, Figure-4D). These data suggest that, in contrast to sedentary ischemia, exercise effectively decreased adipocyte accumulation and enhanced muscle fibers maturation in the regenerating muscle tissue under demand ischemia.

Effect of demand ischemia and exercise on muscle ATP levels

ATP levels were analyzed in the hind limb muscle tissues at the end of sedentary or exercise period. ATP levels were markedly higher in the exercised non-ischemic contralateral muscle when compared to the muscle with demand ischemia (Exercised Contralateral: 17.03 ± 1.97 vs. Demand Ischemia: 10.63 ± 1.10 nmole/mg total protein, $p = 0.015$, Figure-5). There was no difference in the steady state level of ATP between the demand ischemia, sedentary ischemia, and the non-ischemic sedentary contralateral muscles (Sedentary Contralateral:

11.57±1.87 vs. Sedentary Ischemia: 8.45±0.83b nmole/mg total protein, Figure-5). These data suggest that exercise did not potentiate the steady state levels of ATP in the demand ischemia muscle as it did in the contralateral muscle.

Effect of demand ischemia and exercise on UCP-3 and pS473-Akt protein levels

Mitochondrial uncoupling proteins are known to reduce oxidative phosphorylation and decreased membrane potential. UCP-3 protein is highly expressed in the muscle fibers of sedentary ischemia (Figure-6A) compared to demand ischemia (Figure-6B). Quantitative analysis by western blotting showed UCP-3 expression was markedly increased in the muscles that were subjected to sedentary ischemia when compared to sedentary non-ischemic contralateral, demand ischemia, or exercised non-ischemic contralateral muscle (Sedentary Ischemia; 14.7±2.2 vs. Sedentary Contralateral: 7.6±1.7 AU, $p<0.05$, vs. Demand Ischemia: 7.1±1.5 AU, $p<0.01$ and vs. Exercised Contralateral; 6.8±0.6 AU, $p<0.05$, Figure-6C). Furthermore, the pS473-Akt levels were not different between the sedentary and demand ischemia muscles (Sedentary Ischemia: 23.1±2.3 vs. Demand Ischemia: 24.2±2.8 AU, Figure-6D). In contrast, phosphorylated-Akt levels were significantly higher in the exercised non-ischemic contralateral muscle when compared to sedentary non-ischemic contralateral (Exercised Contralateral: 27.1±3.1 vs. Sedentary Contralateral: 16.4±1.6 AU, $p<0.05$, Figure-6D). These findings suggest that exercise improved insulin sensitivity in the non-ischemic muscle in association with augmented ATP levels but it did significantly modulate insulin sensitivity in the demand ischemic muscle.

Discussion

This study demonstrate that exercise enhances indices of autophagy, insulin sensitivity, and energy substrate levels in the non-ischemic contralateral hind limb muscle of obese diabetic mice; however, exercise failed to enhance these markers in demand ischemia muscle. By comparing hind limb muscle morphology of the exercise and the sedentary groups, we observed substantially less adipocyte accumulation and enhanced muscle fiber maturation in demand ischemia compared to sedentary ischemic muscle independent of hind limb perfusion. The hallmark of the autophagy process consists of the recruitment and cleavage of the microtubule-associated protein light chain-3 (LC3) yielding the cytosolic-LC3-I mediated by the cysteine protease (Atg4A) which subsequently is lipidated by binding to phosphatidylethanolamine resulting in the membrane associated LC3-II, which forms dual-membrane cytosolic vesicles called; autophagosome²¹. The ratio of LC3-II/I has been widely used as an index for measuring autophagic influx rate. Our results revealed changes in the LC3 II/I ratio induced only in the exercised non-ischemic contralateral muscle (Figure-2B) while Beclin-1 levels were induced in both the demand ischemia and the non-ischemic contralateral muscles (Figure-2C). We speculate that the autophagy process was hindered in demand ischemia muscle despite the increase in Beclin-1 levels. In obesity and type-2 diabetes, modulation of autophagy is known to be tissue-specific, and the induction of autophagy can be driven by different pathways or proceed independent of certain regulatory proteins under different stress conditions²². Studies in skeletal muscle have indicated that autophagy inhibition or genetic deficiency lead to accumulation of abnormal mitochondria and damaged proteins which disrupt signaling pathways that inhibit protein

synthesis and favor protein degradation leading to atrophy²³. In fact, a study by Kimura et. al showed that Chloroquine treatment elicits deleterious effects on skeletal muscle in experimental model by suppressing autophagy and inducing myopathy²⁴. These negative effects were ameliorated by exercise in animals²⁵. The benefit of exercise on improving skeletal muscle contractility, mitochondrial dysfunction, and insulin sensitivity is well established. Exercise is known to modulate autophagy and the ubiquitin-proteasome pathways activity in healthy and diabetic patients^{26, 27}. Our findings in the exercised non-ischemic contralateral muscle are supported by an animal study that documented an enhanced autophagy and improved mitochondrial dynamics in response to the increase in metabolic demand after exercise²⁸.

Mitochondrial fission and fusion proteins are reported to be upregulated after exercise in obese patients and are proposed as a potential therapeutic target for cardiovascular disease²⁹. Our data show that the protein expression ratios of the mitochondrial dynamic proteins, Opa-1/ Drp-1, were not altered in demand ischemia or in non-ischemic contralateral muscle after the exercise period (Figure-2D). We speculate that this might be due to the shorter exercise period of 4 weeks compared to 12 weeks in the human study, or it may be related to the fact that our animals experiencing a combination of demand ischemia and diabetes during the exercise period. Aberrant muscle regeneration can lead to pathologic adipogenesis in several conditions including ischemia³⁰. As we previously reported, diet induced obese mice demonstrated diminished ATP levels and augmented adipocyte accumulation in the hind limb muscle during the regenerative phase following acute hind limb ischemia compared to non-obese mice¹⁶.

In this report, we demonstrated that hind limb muscle of the obese mice that had unilateral sedentary ischemia had markedly higher adipocyte accumulation while the demand ischemia muscle had significantly reduced adipocyte accumulation after 4 weeks of exercise (Figure-4C). This finding was associated with enhanced muscle fiber maturation indicated by the reduced number of centrally nucleated myofibers in the demand ischemia group (Figure-4D). In sedentary ischemia, we speculate that the differentiation of the regenerating muscle precursors favored adipocyte rather than myoblast differentiation lineage while in demand ischemia this process shifted toward more muscle regeneration and maturation. The exact mechanism of this finding has not been characterized but literature suggests that in order to achieve proper skeletal muscle regeneration, adequate perfusion, efficient inflammatory response, and appropriate satellite cell proliferation and differentiation must be present. Our data exclude the possibility that difference in the recovery of hind limb perfusion following FAL was responsible for the observed differences in adipocyte area between the two groups (Figure-1A).

Skeletal muscle satellite cells are multipotent and capable of entering an alternative mesenchymal differentiation including adipogenesis³¹. The fate of the differentiating satellite cells in mice has been reported to be determined by the transcriptional regulator PRDM16³² which is controlled by an interfering RNA, microRNA-133³³. Further investigation aimed at delineating the mechanism that modulates satellite cell differentiation under demand ischemia need to be explored.

A published report showed evidence of insulin resistance associated with lower skeletal muscle mitochondrial content and reduced mitochondrial proteins including SDHA in the off springs of diabetic patients³⁴. It has been established that mitochondrial proteins content and activity increase following exercise³⁵. Our data showed that SDHA protein level was increased with exercise in both the demand ischemia and the non-ischemic contralateral muscles (Figure-3A). However cytochrome-c was only increased in the non-ischemic exercised contralateral muscle (Figure-3C). This data suggest that the exercise-induced enhancement is diminished in demand ischemia and preserved in the non-ischemic contralateral muscle under diabetes similar to what has been reported by other investigators in diabetic animals with normal limb perfusion³⁶⁻³⁸. ATP production is directly linked to mitochondrial respiration, which was shown to be enhanced following a period endurance exercise³⁹. This is in agreement with our findings in the non-ischemic exercised contralateral limb muscle, which showed higher ATP content compared to sedentary non-ischemic muscle (Figure-5). However, exercise did not enhance ATP levels in the demand ischemia muscle, which also exhibited lower cytochrome-c proteins levels compared to the exercised contralateral limb suggesting that mitochondrial oxidative capacity was not sufficiently enhanced by exercise to generate more energy substrates due to reduced hind limb perfusion (Figure-1A). In diabetes, increased mitochondrial uncoupling is considered a key contributor to reduced contractile efficiency in the cardiac muscle by decreasing mitochondrial oxidative phosphorylation and membrane potential thereby uncoupling ATP synthesis, dissipating energy as heat, and affecting energy metabolism⁴⁰. Our results showed that UCP-3 levels were significantly upregulated in the muscle with sedentary ischemia but not the demand ischemia or non-ischemic contralateral muscle (Figure-6C). However, exercise did not enhance the steady state levels of ATP under demand ischemia as it did in the non-ischemic contralateral muscle (Figure-5). To explain this mismatch between decrease UCP-3 and diminished ATP levels, we speculate that since our assessment of ATP level was performed immediately after the final exercise session, diminished ATP levels may have resulted due to increase demand and inadequate perfusion similar to that of the lower limb muscle in patients with PAD. It will be interesting to verify whether the failure of exercise to augment energy substrate levels in demand ischemia affect hind limb muscle contractility similar to what we observed in our previous findings regarding acute hind limb ischemia reperfusion injury in diabetic mice¹⁶.

To date, obesity and claudication are mainly treated by medical measures consisting of dietary restriction, glycemic control, smoking cessation, and exercise while surgery is reserved for advanced cases. This treatment strategy is only partially successful, and therefore interest to develop additional therapeutic adjuncts exists. To achieve this, an understanding of the molecular mechanisms involved in the coordination of mitochondrial biogenesis and autophagy that will delineate methods and intervention to manipulate them will be critical. Several pharmacological approaches to enhance mitochondrial function and biogenesis, energy homeostasis have been suggested in cardiovascular disease and diabetes. They included AMPK activators⁴¹ or incretins enhancers³⁶, which might also be useful for diabetic patients with PAD.

Conclusions

Our results demonstrated that exercising diabetic obese mice with unilateral hind limb demand ischemia suppressed adipocyte accumulation and enhanced myofiber maturation compared to sedentary mice. The morphologic changes were not associated with enhanced indices of autophagy, mitochondrial biogenesis or energy substrate levels at the end of the exercise period suggesting that exercise alone is not sufficient to modulate these important biological processes in the setting of demand ischemia. Future studies aimed at assessing muscle function and testing pharmacologic mediators that enhance autophagy and mitochondrial function may be useful to improve the prognosis of diabetes associated PAD.

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Figure-1. Effect of daily treadmill exercise on hind limb perfusion and body weight

Image A is a summary of laser Doppler scanning expressed as the perfusion ratio (flux unit ratio) of the ischemia to the contralateral hind limb in each mouse at baseline, immediately after FAL, and then once a week thereafter during the stabilization period and the subsequent exercise or sedentary period. Both groups showed a remarkable drop in hind limb perfusion after surgery and partial recovery over the following weeks after surgery (*, + $p < 0.00001$, A). There was no difference in hind limb perfusion between the exercised and sedentary groups at any time point while hind limb perfusion remained significantly lower than the respective starting baseline perfusion at the end of 6 weeks. In addition, the sedentary group gained markedly more weight over the course of the 4-week experimental period compared to the exercised mice (B, * $p < 0.0001$). These data indicate that exercise prevented additional increase in body weight but it did not alter hind limb perfusion in the obese mice.

Figure-2. Effect of demand or sedentary ischemia on autophagy in hind limb skeletal muscle
Representative immunoblots of muscle tissue lysate for markers of autophagy; LC3B-II/I, Beclin-1 and mitochondrial biogenesis regulating proteins; OPA-1 and DRP-1. Graphical presentation of the densitometric quantification of immunoblots from 7–8 mice samples for each group is summarized in graphs. Graph (B) demonstrates that the expression ratio of lipidated LC3B-II to the non-lipidated LC3B-I (LC3B-II/I) in the exercised contralateral muscle was significantly higher than the sedentary contralateral muscle (B, * $p < 0.05$) and sedentary ischemic muscle (B, ** $p < 0.01$) indicating a higher rate of autophagy, but there was no difference between the demand ischemia and the sedentary contralateral muscle (B). In contrast, Beclin-1 levels were significantly higher in both the exercised contralateral (C, * $p < 0.01$) and the demand ischemia muscle (Graph C, ** $p < 0.01$) compared to sedentary contralateral muscle. These data suggest that exercise upregulated macroautophagy in the tissue with normal limb perfusion, while enhancing Beclin-1 in both contralateral and ischemic muscle. The expression ratio of the mitochondrial fusion protein, Opa-1, to the mitochondrial fission protein, Drp-1, revealed no differences between the groups (Graph D).

Figure 3. Effect of demand ischemia and exercise on the levels of mitochondrial proteins
 Panel A contains representative western blotting analysis of the mitochondrial complex II associated protein (SDHA) and cytochrome-c. Results summary of SDHA protein expression showed significantly higher levels of SDHA in the exercised contralateral (B, * $p < 0.05$) and demand ischemia muscle (B, ** $p < 0.01$) compared to sedentary contralateral. However, cytochrome-c levels were significantly higher only in the exercised contralateral muscles (C, * $p < 0.05$) compared to sedentary contralateral muscle while demand ischemia did not change. These data suggest that exercise enhances the level of mitochondrial proteins in the muscle with normal perfusion while it fails to increase selected mitochondrial proteins under demand ischemia condition.

Figure-4. Effect of demand ischemia and exercise on skeletal muscle tissue morphology

Representative images A and B illustrate the histopathology of the TA muscle cross section showing extensive adipocyte accumulation (black arrows) among the regenerating myofibers in sedentary ischemia (black arrows, A), while less fat cells were observed in the tissue subjected to demand ischemia (black arrows, B). Quantitative analysis revealed markedly less average fat cell area under demand ischemia compared to sedentary ischemia, (*p=0.016, summary graph, C). The myofibers presented with nuclei in the center indicate immature fiber (yellow arrows in images A&B). Exercise significantly reduced the percentage of myofibers with central nuclei in the demand ischemia compared to sedentary ischemia muscle indicating an enhanced skeletal muscle differentiation (*p=0.005, summary graph, C).

Figure-5. Effect of demand ischemia and exercise on muscle ATP levels

Chemiluminescence analysis of ATP levels in hind limb muscle tissue showed significantly higher ATP in the exercised contralateral hind limb compared to demand ischemia (* $p=0.015$, D). There was no difference in the steady state level of ATP between the demand ischemia, sedentary ischemia, and sedentary contralateral hind limb muscles.

Figure-6. Effect of demand ischemia and exercise on UCP-3 and phosphorylated-Akt protein expression

Images A&B are representative chromogenic immunohistochemistry detections of UCP-3 protein in the TA muscle tissue from sedentary and demand ischemia conditions. Sedentary ischemia muscle (A) revealed extensive UCP-3 staining (brown color) inside the myofibers, together with several adipocytes in the field (black arrow). In contrast, demand Ischemia (B) showed considerably less UCP-3 staining and extensive adipocyte infiltration (black arrows). Western blot analysis of the UCP-3 (C) showed marked increase levels in the sedentary ischemia compared to exercise contralateral (** $p < 0.01$), sedentary contralateral (* $p < 0.05$) or demand ischemia muscles (** $p < 0.01$). Western blot analysis of phosphorylated-Akt, showed markedly enhanced pS473-Akt levels only in the exercised contralateral muscle compared to sedentary contralateral (* $p < 0.05$, D).