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Revascularization and muscle adaptation to limb demand ischemia in diet-induced obese mice

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ABSTRACT

Background: Obesity and type 2 diabetes are major risk factors for peripheral arterial disease in humans, which can result in lower limb demand ischemia and exercise intolerance. Exercise triggers skeletal muscle adaptation including increased vasculogenesis. The goal of this study was to determine whether demand ischemia modulates revascularization, fiber size, and signaling pathways in the ischemic hind limb muscles of mice with diet-induced obesity (DIO).

Materials and methods: DIO mice ($n = 7$) underwent unilateral femoral artery ligation and recovered for 2 wks followed by 4 wks with daily treadmill exercise to induce demand ischemia. A parallel sedentary ischemia (SI) group ($n = 7$) had femoral artery ligation without exercise. The contralateral limb muscles of SI served as control. Muscles were examined for capillary density, myofiber cross-sectional area, cytokine levels, and phosphorylation of STAT3 and ERK1/2.

Results: Exercise significantly enhanced capillary density ($P < 0.01$) and markedly lowered cross-sectional area ($P < 0.001$) in demand ischemia compared with SI. These findings coincided with a significant increase in granulocyte colony-stimulating factor ($P < 0.001$) and interleukin-7 ($P < 0.01$) levels. In addition, phosphorylation levels of STAT3 and ERK1/2 ($P < 0.01$) were increased, whereas UCP1 and monocyte chemoattractant protein-1 protein levels were lower ($P < 0.05$) without altering vascular endothelial growth factor and tumor necrosis factor alpha protein levels. Demand ischemia increased the *PGC1α* messenger RNA ($P < 0.001$) without augmenting PGC1α protein levels.

Conclusions: Exercise-induced limb demand ischemia in the setting of DIO causes myofiber atrophy despite an increase in muscle capillary density. The combination of persistent

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increase in tumor necrosis factor alpha, lower vascular endothelial growth factor, and failure to increase PGC1α protein may reflect a deficient adaption to demand ischemia in DIO.

Introduction

Diabetes, obesity, and metabolic syndrome increase the risk of peripheral arterial disease (PAD).[1,2] In PAD patients, muscle degeneration as well as vascular and mitochondrial dysfunctions are associated with evidence of increased inflammation[3] and oxidative stress, which may contribute to limb dysfunction.[4] In patients with diabetes or the metabolic syndrome, muscle tissue remodeling becomes defective due to the deleterious microenvironment manifested by muscle degeneration, fatty infiltration, inflammation, and diminished angiogenic capacity.[5] Arterial stenosis limits adequate perfusion needed by increased workload on the limb muscles during ambulation, thereby creating demand ischemia, which clinically manifest intermittent claudication or exercise intolerance.[6] PAD can progress in severity to greatly limiting mobility and, in advanced cases, may lead to rest pain, critical limb ischemia and potentially tissue loss.[7]

To date, current treatments for PAD patients with stable intermittent claudication have been centered on reducing cardiovascular disease risk factors and regaining limb function through exercise.[7] Meanwhile, surgical interventions are reserved for severe functional disability, rest pain, or tissue loss.[7] Recent literature suggested that the optimal exercise program that will avoid potential adverse effects in PAD patients has not been well characterized,[8] and the degree of benefit from exercise can be variable.[9]

To simulate PAD in humans, our laboratory investigated the effect of chronic hind limb ischemia using a murine model of diet-induced obesity (DIO) under sedentary or exercise-induced demand ischemia conditions.[10] Using this model, we demonstrated that chronic sedentary ischemia (SI) causes diminished energy metabolism associated with increased adipocyte infiltration and increases expression of uncoupling protein-3 (UCP-3) protein in the hind limb muscles, indicating mitochondrial uncoupling and defective energy metabolism.[10] Subsequent analysis of a parallel group of mice that were subjected to exercise-induced limb demand ischemia showed reduced adipocyte infiltration and decreased levels of mitochondrial uncoupling protein, UCP-3, without an improvement in metabolic substrate levels, indicating persistent metabolic deficit. Furthermore, exercise did not enhance hind limb perfusion in the ischemic limb of DIO mice as it was assessed by laser Doppler imaging, which reports on gross perfusion of the hind limb. However, in that study, muscle fiber size and vascular density were not assessed.[9]

In the present study, we therefore aimed to compare capillary density and changes in myofiber size in response to demand or SI in the hind limbs of DIO mice. We hypothesized that exercise-induced limb demand ischemia in the presence of metabolic deficit would promote differential proangiogenic and inflammatory responses and modulate muscle myofiber size compared with sedentary hind limb ischemia of DIO mice.

Accordingly, hind limb skeletal muscle tissues were assessed for capillary density and myofiber cross-sectional area (CSA) as well as the protein content of proangiogenic growth factors and cytokines, which are known to be modulated in PAD patients and to mediate muscle revascularization and remodeling.[11-14] Phosphorylation of the signaling molecules, ERK1/2 and STAT3, was evaluated due to their involvement in cellular growth, stress adaptation, cytokine responses, and survival.[15,16] In addition, the expression level of UCP1 was assessed because it will report on infiltrating adipocyte differentiation, which was reported to decrease under demand ischemia. Finally, the expression level of *PGC1α* gene was also assessed as it is known to regulate muscle adaptation to exercise-induced metabolic demand[17,18] and several pathways associated with angiogenesis[19] and cytokine release.[20]

Materials and methods

Animal protocol

Animal care and experimental procedures complied 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 were approved by the Institutional Animal Care and Use Committee at the Massachusetts General Hospital. Age-matched male C57BL6 mice were acquired from Jackson Laboratory (Bar Harbor, ME) and were continuously fed with high-fat diet consisting of 60% kcal derived from fat for 26 wks to induce obesity, insulin resistance, and diabetes.[21] Male DIO mice were enrolled in this study because they are more susceptible to developing metabolic syndrome and diabetes compared with female mice.[22-24] To induce hind limb ischemia, all mice were subjected to unilateral femoral artery ligation (FAL) surgery and were then allowed to recover for 2 wks as previously described.[10] Subsequently, mice were divided into two groups: an exercise-induced demand ischemia group and a sedentary ischemia (SI) group. The demand ischemia group ($n = 7$) underwent 60 min of daily treadmill exercise (12 m/min speed at 10° incline) for 4 wks to induce demand ischemia in the hind limb that underwent FAL. The SI group that had FAL was not exercised for 4 wks after the 2-wks recovery period generating SI condition in the hind limb muscle ($n = 7$). For molecular and histologic analyses, the nonischemic contralateral hind limb muscles that were harvested from the sedentary group served as baseline control ($n = 7$). Hind limb tissues were harvested at the end of 4 wks of exercise or sedentary periods and divided for histologic evaluation or stored at −80°C for molecular analyses.

Histologic measurement of hind limb muscle fiber cross-sectional area

To determine the changes in myofiber size, tissues were dehydrated in graded acetone and embedded in JB-4 polymer (Polysciences Inc, Warrington, PA). Next, 2-μm thick cross sections were obtained from the mid belly of the tibialis