Diminished force production and mitochondrial respiratory deficits are strain-dependent myopathies of subacute limb ischemia

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Objective: **Reduced skeletal muscle mitochondrial function might be a contributing mechanism to the myopathy and activity based limitations that typically plague patients with peripheral arterial disease (PAD). We hypothesized that mitochondrial dysfunction, myofiber atrophy, and muscle contractile deficits are inherently determined by the genetic background of regenerating ischemic mouse skeletal muscle, similar to how patient genetics affect the distribution of disease severity with clinical PAD.**

Methods: **Genetically ischemia protected (C57BL/6) and susceptible (BALB/c) mice underwent either unilateral subacute hind limb ischemia (SLI) or myotoxic injury (cardiotoxin) for 28 days. Limbs were monitored for blood flow and tissue oxygen saturation and tissue was collected for the assessment of histology, muscle contractile force, gene expression, mitochondrial content, and respiratory function.**

Results: **Despite similar tissue O_2 saturation and mitochondrial content between strains, BALB/c mice suffered persistent ischemic myofiber atrophy (55.3% of C57BL/6) and muscle contractile deficits (approximately 25% of C57BL/6 across multiple stimulation frequencies). SLI also reduced BALB/c mitochondrial respiratory capacity, assessed in either isolated mitochondria (58.3% of C57BL/6 at SLI on day (d)7, 59.1% of C57BL/6 at SLI d28 across multiple conditions) or permeabilized myofibers (38.9% of C57BL/6 at SLI d7; 76.2% of C57BL/6 at SLI d28 across multiple conditions). SLI also resulted in decreased calcium retention capacity (56.0% of C57BL/6) in BALB/c mitochondria. Nonischemic cardiotoxin injury revealed similar recovery of myofiber area, contractile force, mitochondrial respiratory capacity, and calcium retention between strains.**

Conclusions: **Ischemia-susceptible BALB/c mice suffered persistent muscle atrophy, impaired muscle function, and mitochondrial respiratory deficits during SLI. Interestingly, parental strain susceptibility to myopathy appears specific to regenerative insults including an ischemic component. Our findings indicate that the functional deficits that plague PAD patients could include mitochondrial respiratory deficits genetically inherent to the regenerating muscle myofibers. (J Vasc Surg 2016;■:1-11.)**

Clinical Relevance: **Skeletal muscle morphology and function are key predictors of clinical manifestation and outcomes in peripheral arterial disease. Our findings show that genetic background is a critical determinant of muscle functional deficits and mitochondrial respiration. Because of the range of peripheral arterial disease manifestations, BALB/c mice provide a useful model for studying the role of muscle and mitochondrial respiratory functional abnormalities in determining morbidity and mortality outcomes in genetically susceptible patients. Novel therapies that directly target the muscle tissue response to limb ischemia could be used alone or in conjunction with current revascularization therapies to reduce morbidity and mortality outcomes in claudicants or patients with critical limb ischemia.**

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The recent identification of differences in the clinical course of intermittent claudication (pain with exertion that is relieved with rest) and critical limb ischemia (CLI, pain at rest with or without tissue necrosis or gangrene) raise the intriguing possibility that these represent genetically determined and distinct phenotypic manifestations of peripheral arterial disease (PAD).[1-7] Preclinically, different inbred mouse strains have dramatically different responses to a murine model of limb ischemia, analogous to the range of responses seen in human patients. For example, BALB/c mice show substantial muscle necrosis after subacute and acute models of ischemia similar to the myopathy observed in patients with CLI, whereas C57BL/6 mice recover rapidly from ischemia without substantial tissue loss or myopathy.[2,3,8,9] Genetic haplotype analysis in these mice identified a quantitative trait locus associated with tissue necrosis that contained 37 genes with no known role in vascular biology.[3] The findings from these studies implies that genetic susceptibility to limb ischemia might be a key factor in the pathology of PAD/CLI. The possibility of genetic regulation of ischemic limb (L) pathology is further highlighted by the underwhelming results of angiogenic/neovascularization clinical trials[10] in PAD patients.

Patients with PAD, in addition to vascular defects, have altered muscle metabolism, mitochondrial respiration, expression of mitochondrial enzymes, increased oxidative stress, and somatic mutations in mitochondrial genes.[11-17] This implies that reduced skeletal muscle mitochondrial function might be a contributing mechanism to the myopathy and activity based limitations that typically plague these patients. We hypothesized that mitochondrial dysfunction, myofiber atrophy, and contractile deficits are inherently determined by the genetic background of the murine regenerating ischemic skeletal muscle. Our findings indicated that myopathy associated with subacute ischemia uniquely involves mitochondrial functional abnormalities that parallel deficits in muscle function and regeneration. Although the functional deficits that plague PAD patients might initially be caused by occlusive obstruction of blood flow to the limb, the inability to recover muscle mass and function might involve analogous mitochondrial respiratory deficits genetically inherent to the muscle myofibers.

METHODS

Detailed information is shown in the Appendix (online only).

Animals. Experiments were conducted on adult (aged 12-16 week) C57BL/6J (n = 33) or BALB/cJ (n = 36) mice. All work was approved by the Institutional Review Committee of East Carolina University and complied with the *Guide for the Care and Use of Laboratory Animals*.[18] Subacute ischemia was performed as previously described.[9] The cardiotoxin (CTX) model of mouse muscle regeneration was performed as previously described[19] using intramuscular injections of *Naja nigricollis* venom.

Assessment of limb perfusion and O_2 saturation of tissue. Limb blood flow was measured using laser Doppler perfusion imaging (LDPI) as previously described.[9] Tissue oxygen saturation (SO_2) was assessed using a moorVMS-OXY white light spectrometer with a CPT-300 optical probe (Moor Instruments, Devon, United Kingdom).

Immunofluorescence and histological analysis. Skeletal muscle morphology, lipid and collagen deposition, vessel density, and muscle myofiber phenotype were assessed using standard light microscopy and immunofluorescence (IF). IF and microscopy muscle regeneration was assessed using magnification ×40 tiled hematoxylin and eosin (H&E) images using an Aperio CS2 digital slide analyzer (Leica Biosystems, Buffalo Grove, Ill). Lipid droplet content was assessed using Oil Red-O counterstained in Mayer hematoxylin, as well as IF for dystrophin and boron-dipyrromethene (BODIPY) 493/503 (Thermo Fisher Scientific, Waltham, Mass). Collagen deposition was visualized using staining in Weigert hematoxylin solution and picrosirius red (PR). Vascular density was assessed using IF as previously described[8,9] and is presented as the mean CD31-positive ($CD31^+$) area per magnified ×20 field of view. Muscle fiber typing was performed as previously described[20] using IF and presented as a proportion of total myofibers of each fiber type (myosin heavy chain [MHC] type IIa, MyHC type IIb, or MyHC type IIx).

Immunoblot analysis. Western blotting was performed using standard methods. Blots were visualized with chemiluminescence using standard film procedures.

Preparation of isolated skeletal muscle mitochondria for respirometry. Skeletal muscle mitochondria were isolated from the gastrocnemius, plantaris, and soleus muscles as described.[21] High-resolution O_2 consumption measurements were conducted using the OROBOROS O2K Oxygraph (Oroboros Instruments Corp, Innsbruck, Austria). The rate of respiration was expressed as pmol/s/mg mitochondrial protein.

Preparation of permeabilized muscle fibers for myofiber respiration. A portion of the red gastrocnemius muscle was removed and fiber bundles were separated along their longitudinal axis as previously described.[22] The rate of respiration was normalized to the myofiber dry weight and expressed as pmol/s/mg dry weight.

Mitochondrial calcium retention capacity. Changes in extramitochondrial calcium concentration were monitored fluorometrically using Calcium Green (1 μM, excitation/emission 506/532 nm, Invitrogen, Waltham, Mass) as per manufacturer's instructions.

Citrate synthase activity assays. Activity assays were performed using a citrate synthase activity assay kit (Sigma-Aldrich, St. Louis, Mo) as per manufacturer's instructions.

Muscle contractile force measurements. Contractile force measurements were performed using isolated extensor digitorum longus (EDL) muscles as previously described.[23]

Total RNA and quantitative reverse transcription-polymerase chain reaction gene expression analysis. Total RNA was extracted from mouse EDL muscles using TRIzol (Invitrogen) and reverse-transcribed using SuperScript IV (Invitrogen). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using an ABI ViiA-7 system (Applied Biosystems, Waltham, Mass).

Statistics. Data are presented as a ratio of the treated (ischemic or CTX injected) left (L) to the untreated (non-ischemic or saline sham injected) contralateral control (R) limb and mean ± standard error of the mean. Force frequency and fiber phenotype data are presented as L and R for each strain with mean ± standard error of the mean. Statistical analyses were carried out using StatPlus:mac (version 2009; AnalystSoft, Walnut, Calif), Vassarstats (www.vassarstats.net), or Prism 6 (version 6.0d; Graphpad Software Inc, La Jolla, Calif) software. Two sided *t*-tests were performed a priori to determine mean differences in the control limbs of the strains. All other data were compared using Student two-tailed *t*-test or analysis of