APPENDIX I. SUPPLEMENTAL MATERIAL

Subacute Limb Ischemia Induces Skeletal Muscle Injury in Genetically Susceptible Mice Independent of Vascular Density

Short Title: Subacute limb ischemia induces muscle injury

Joseph M. McClung[1,2,#,] Timothy J. McCord[3], Kevin Southerland[4], Cameron A. Schmidt[1,2], Michael E. Padgett[3], Terence E. Ryan[1,2], and Christopher D. Kontos[3,5]

[1]Department of Physiology and [2]Diabetes and Obesity Institute, East Carolina University, Brody Medical Center, Greenville, NC.

[3]Division of Cardiology, Department of Medicine; [4]Division of General Surgery, Department of Surgery; [5]Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC.

#Correspondence should be addressed to:

JMM: Diabetes and Obesity Institute, Office #4109, Mail Stop 743, East Carolina Heart Institute, Brody School of Medicine at East Carolina University, 115 Heart Drive, Greenville, NC 27834-4354. Tel: 252-737-5034 (office); Fax: 252-744-3460; email: mcclungj@ecu.edu

Materials and Methods

Animals. Experiments were conducted on adult C57/BL6 (N=51) or BALB/c (N=58). All mice were ≥12 weeks old the study was approved by the Institutional Review Committees of Duke University Medical Center or East Carolina University. All animal care complied with the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Washington: National Academy Press, 1996.

Animal Models of Ischemic Peripheral Artery Disease. Mice were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). Acute hindlimb ischemia (ALI)[1] was induced by ligation and transection of the femoral artery proximally inferior to the inguinal ligament and immediately proximal to the superficial caudal epigastric artery, and distally immediately proximal to the bifurcation of the popliteal and saphenous arteries. Subacute limb ischemia (SLI) was performed by placement of ameroid constrictors (ACs) on the femoral artery as described further below. The ameroid constrictors (0.25mm internal diameter, Research Instruments SW, Escondido, CA) have been described previously [2, 3] and consist of a stainless steel sheath surrounding a hygroscopic casein ring. ACs were sterilized with ethylene gas, and were used to induce ischemia in one of two ways: 1) An AC was placed on both the proximal and distal femoral artery (2AC model) in a modification of the approach described previously by Yang et al [3]. The proximal ameroid constrictor was placed around the femoral artery, inferior to the inguinal ligament and immediately proximal to the superficial caudal epigastric artery, and the distal ameroid constrictor was placed immediately proximal to the bifurcation of the popliteal and saphenous arteries; 2) A single AC was placed on the femoral artery immediately proximal to the origin of the superficial caudal epigastric artery (1AC model). To minimize the potential

1 for acute ischemia, the inferior epigastric, lateral circumflex, and superficial epigastric artery
2 branches of the femoral artery were left intact in all models (ALI, 2AC, 1AC), thereby
3 preserving collateral perfusion to the limb. Mice were closely monitored during the postoperative
4 period, and perfusion in the ischemic and contralateral non-ischemic limbs was measured
5 immediately after surgery and at the indicated times afterwards up to 4 weeks post-operatively.
6 The extent of necrosis, if any, in ischemic limbs was recorded post-operatively using the
7 previously described semi-quantitative scale [1]: grade 0, no necrosis in ischemic limb; grade I,
8 necrosis limited to toes; grade II, necrosis extending to dorsum pedis; grade III, necrosis
9 extending to crus; and grade IV, necrosis extending to mid-tibia or complete limb necrosis. For
10 limb necrosis, each animal is scored by a blinded investigator at each time point and all scores
11 are assigned across each model by the same blinded investigator.
12 *Assessment of Limb Perfusion.* To determine blood flow recovery after AC-induced subacute
13 femoral artery occlusion, laser Doppler perfusion image (LDPI) scanning was performed on
14 animals placed on a warming pad in the supine position after anesthesia, using a Moor
15 Instruments LDI2-High Resolution (830nM) System (Moor, Axminster, UK) up to 28 days
16 postoperatively. Images were analyzed with the MoorLDI™ Image Review software. Results
17 were expressed as the ratio of perfusion in the ischemic to the non-ischemic hind limb/paw.
18 *Primary Antibodies and Materials.* The following commercial antibodies were used: tubulin
19 (Santa Cruz), CD31 (AbdSerotec MCA-1364), SMA (DAKO, 1A4), dystrophin (Thermo
20 Scientific RB-9024), dystrophin (MANDYS1 3B7, Developmental Studies Hybridoma Bank,
21 Iowa City, IA), and macrophages (Mac-1: MCA519G, Serotec, Raleigh, NC).
22 *Histological Analysis.* Skeletal muscle morphology, vessel density, and markers of muscle
23 regeneration were assessed by standard light microscopy and immunofluorescence microscopy.

1 The femoral artery around AC placement was isolated at sacrifice and frozen in liquid nitrogen
2 cooled isopentane for sectioning. Eight-μm-thick transverse sections were cut from mouse TA
3 muscle, frozen in liquid nitrogen-cooled isopentane in optimum cutting temperature (OCT)
4 medium. Sections were allowed to come to room temperature and were either stained with
5 hematoxylin and eosin using standard methods or fixed and permeabilized with ice-cold acetone
6 for 10 min at 4°C. Fixed sections were allowed to air dry for 5 min at room temperature (RT)
7 and rehydrated in 1× PBS before blocking in 5% normal goat serum (Sigma) in 1× PBS at RT for
8 45 min. Slides were then incubated overnight at 4°C in a primary antibody solution. Slides were
9 then washed 3× in 1× PBS at RT and incubated for 1h at RT in the dark in a secondary solution
10 containing a 1:250 dilution of Alexa Fluor 488-, 568-, or 633-conjugated secondary antibodies in
11 blocking solution. Sections were then washed in the dark 3× for 5 min each with 1× PBS at RT,
12 and slipcovers were mounted using Vectashield HardSet Mounting Medium with DAPI (Vector
13 Labs H-1500). Images were captured using a Zeiss Axio Observer Inverted Laser Scanning
14 Microscope (LSM) 510 utilizing the Zeiss LSM 510 software (v. 4.2) and analyzed by a blinded
15 investigator using ImageJ software (NIH, v. 1.47v). Vessel density was expressed as the number
16 of $CD31^+$ or SMA^+ cells per μm^2 of muscle analyzed. The density of $CD31^+$ vessels was
17 quantified as an indicator of capillary density changes in the distal limb muscle and represents
18 capillary regression or angiogenesis. The size of SMA^+ vessels was also determined and
19 expressed as the percentage of SMA^+ vessels per total muscle area (in μm^2) analyzed. The size
20 and density of SMA^+ vessels was used for the verification of changes in intermediate or arteriole
21 density in the distal ischemic limb muscle. Macrophage immunostaining was quantified as the
22 percentage of total TA muscle area Mac-1^+ by a blinded investigator using ImageJ software
23 (NIH, v. 1.47v)

Limb Muscle Morphology and Regeneration. Sections from muscle samples were stained with H&E, and digital images were obtained at ×20 magnification for the analysis of non-contractile tissue (NCT). A 528 (22×24)-point grid was overlaid on 3 images from each animal, and points were analyzed for overlap with myofibers or outside of myofibers. NCT was expressed as the percentage of points overlapping non-myofiber area divided by the number of points overlapping myofibers. Total myofiber cross sectional area (CSA, μm^2) was determined from ×20 images by tracing the area of approximately 300 individual fibers per animal with NIH ImageJ image analysis software. The same images were also utilized for the quantification of centralized myofiber nuclei, which were expressed as a percentage (%) of total myofibers in the same image.

Statistical Analysis. Statistical analyses were carried out using StatPlus:mac (v. 2009) statistical analysis software, Vassarstats (www.vassarstats.net) or Prism 6 (v. 6.0d). Non-parametric necrosis score data are presented as proportions of mice falling within each necrosis score criteria for each strain and day. Necrosis scores between strains were compared using Mann-Whitney U tests. Fiber size distributions were compared with the χ^2 test. All other data were compared using ANOVA and Student's 2-tailed *t*-test to identify strain dependent differences in outcome measures and within strain differences in surgical limbs. In all cases, $P < 0.05$ was considered statistically significant.

References:

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