Supplemental Materials and Methods:

Protein Analysis.

Total cellular protein was extracted and protein concentrations were determined using Bradford Assay. Protein extracts (30-50 μg) were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with the following antibodies: rabbit polyclonal anti-phospho-eNOS Ser1177 (1:500), rabbit polyclonal anti-eNOS (1:1000) (Cell Signaling, Danville, MA), and mouse monoclonal anti-actin (1:2000) (Sigma-Aldrich, St Louis, MO), rabbit polyclonal anti-human myoglobin (Dako, Carpinteria, CA). After incubation with approprioate secondary antibodies conjugated to horse-radish peroxidase (1:5000, Santa Cruz biotechnology, CA), immune reactivity was detected using chemiluminescence. Results were quantified by densitometry using Image J 1.37, NIH, USA. VEGF-A concentrations were determined using the Quantikine Mouse VEGF-A Immunoassay (R&D systems, MN), following the manufacturer's recommendations. Each sample was assayed in duplicate and the values were normalized to total protein concentration. Myoglobin was quantified using a mouse myoglobin ELISA kit (Life Diagnostics, West Chester, PA), following manufacturer's recommendations.

Measures of Vessel Density, Apoptosis, Macrophage Number, in Muscles.

For determination of capillary density, endothelial cells were identified by immunohistochemical staining using rat anti-CD31 antibody (1:200 dilution, Serotec, Raleigh, NC). Endothelial cells were counted in six random high-power (200×) fields from each ischemic and non-ischemic limb muscles, and expressed as the number of CD31 positive cells per fiber. Apoptosis in skeletal muscle was detected using TUNEL staining (ApopTag In Situ kit, Chemicon, Temecula, CA) and Caspase-3 Activity Assay (Biovision, Mountain View, CA). The apoptotic index was expressed as the TUNEL positive nuclei as a percent of the total number of counted nuclei. The count was done on 3 random fields (200×) per section. For assessment of macrophage infiltration, immunohistochemistry was done on frozen sections from ischemic tissue using rat anti mouse Cd11b (Mac-1) antibody at 1:150 dilution (BD Pharmigen, San Diego, CA). Immune reactivity was detected using an alkaline phosphatase conjugated anti-rabbit secondary kit (Vector Labs, Burlingame, CA). Sections were then counterstained with Hematoxylin.

For quantitation of arterioles, double labeled immunoflorescence was done using rat anti-CD31 antibody at1:25 dilution (Serotec, Raleigh, NC) and mouse monoclonal anti-alpha smooth muscle actin (Sigma-Aldrich, St. Louis, MO) at 1:500 dilution. FITC-conjugated and Rhodamine-conjugated secondaries were used to detect CD31 and

smooth muscle actin respectively. Sections incubated with nonspecific IgGs were used as negative controls. The number of microvessels that stained double positive were then counted and expressed as vessel number per high power field (400X).

Measurement of Nitric Oxide Synthase (NOS) Activity.

NOS activity was measured in frozen sections from aortas and 14-day ischemic muscle using the principle of conversion of non-fluorescent reagent, 4,5-diaminofluorescein (DAF-2DA from Cell Technology Inc, Mountain View, CA) to fluorescent DAF-2T in presence of NO. Following incubation of samples with DAF-2DA, L-Arginine (1mM) is applied to the samples, which leads to production of NO. NO then reacts with DAF-2DA to form fluorescent DAF-2T. Development of fluorescence was monitored until 40 minutes, and used as an index of NOS activity. To ensure that the signal is NO derived, after DAF-2DA incubation, control sections were incubated with 10mM L-NAME . The integrated image densities were quantified using Image J (Image J 1.38, NIH, MD).

Measurement of Tissue Levels of cGMP.

Total protein was extracted using Tris-HCl buffer from the Gastrocnemius muscle and pieces of aorta from MbTg and Wt mice. cGMP was measured in the protein extracts using Parameter cGMPAssay (R &D Systems, Minneapolis, MN), following manufacturer's instructions. Level of cGMP in each sample was normalized to the total protein concentration.

Measurement of Vascular Reactivity.

We studied aortic rings to investigate whether myoglobin over-expression altered endothelium dependent and endothelium independent relaxation responses. Five mm rings from the aortas were placed in physiological saline solution [PSS; NaCl 140.0 mM, KCl 5.0 mM, $CaCl_2$ 1.6 mM, $MgSO_4$ 1.2mM, 3-[N-morpholino]-propane sulfonic acid (MOPS)] at 4°C. Aortic rings were then suspended between an isometric force transducer and length positioning support post of a Radnoti myograph system (ADI instruments, Colorado Spring, CO), bathed in PSS at 37°C and gassed with 95% Oxygen and 5% Carbon-dioxide. A resting tension of 160 mg was applied to the rings for 30 minutes for equilibration. Following equilibration, rings were stimulated with 100 mM K+PSS solution for 10 minutes to test viability. Rings were then rinsed with PSS until force returned to passive tension level. Cumulative dose response curves for phenylephrine (PE, 0.01-30 mM) were done, and the rings were washed for 60 minutes before examining relaxation response of 30uMPE pre-constricted tissues to either acetylcholine (ACh,

0.001-30 μM), or sodium nitroprusside (SNP) (0.001-30 μM). The effective dose (ED_{50}) was calculated from a four parameter logistic fit of the mean. The maximum active force generation in response to 100mM K+ and 30μM PE was normalized to the cross sectional area of the aortic rings.

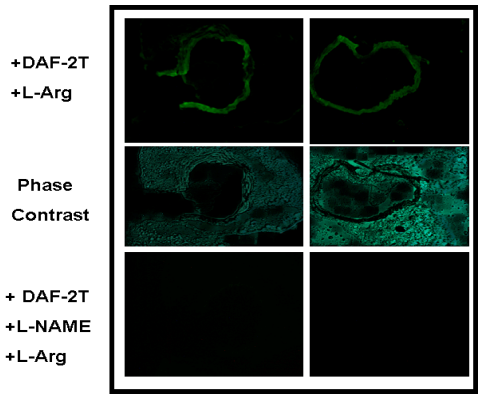
Immunohistochemical Detection of Myoglobin.

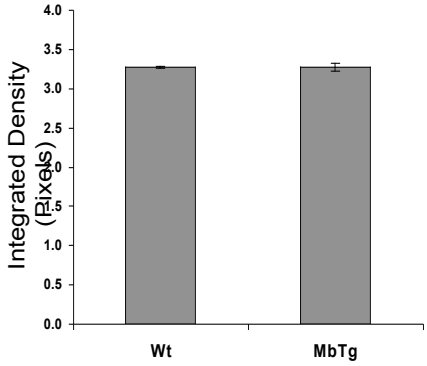
We examined for expression of myoglobin in vascular smooth muscle using immunohistochemistry. Cross sections of skeletal muscle were used as positive controls. Frozen sections from aorta and tibialis anterior muscle were incubated with 1:500 dilution of rabbit anti-human myoglobin antibody (Biocare Medical, Concord, CA) for 1 hour at room temperature. Immune reactivity was detected using an alkaline phosphatase conjugated anti-rabbit secondary kit (Vector Labs, Burlingame, CA). Sections were then counterstained with Hematoxylin.

Supplemental Figure 1.

A. Frozen sections of aorta from MbTg and Wt mice (n=5/group) show no difference in NOS activity
B. Levels of cGMP in aortas (n=4/group, P=NS). There was no difference in the cGMP levels in aorta between MbTg and Wt mice.

A.





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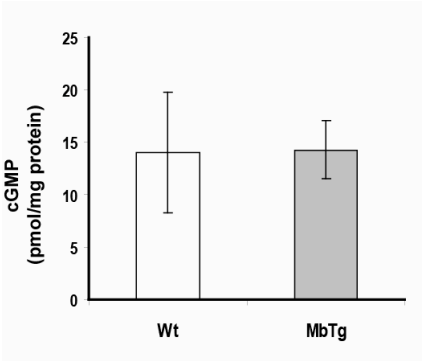
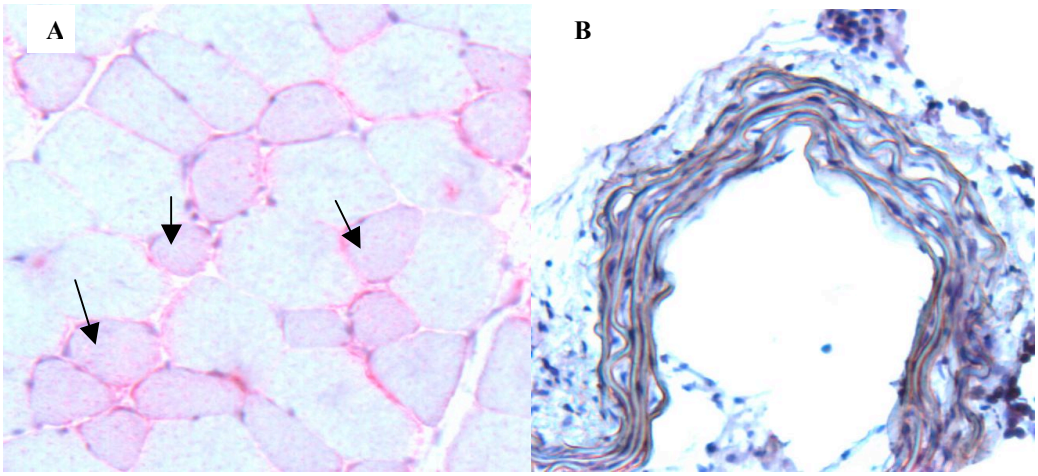


Supplemental Figure 2.

A. Cross section of skeletal muscle (Tibialis anterior), showing myoglobin expression, primarily in the smaller, oxidative myofibers. **B.** Cross section of aorta. Smooth muscle layer does not stain positive for myoglobin.



Supplemental Figure 3.

A. There was no difference in macrophage count in the ischemic tissue of Wt and MbTg mice (n=4/group, 3 sections per mouse).
B. There was no difference in the number of CD31 and smooth muscle double positive vessels in the ischemic tissue of Wt and MbTg mice (n=6/group).