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Myocyte Specific Overexpression of Myoglobin Impairs Angiogenesis After Hind-Limb Ischemia

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**Objective**—In preclinical models of peripheral arterial disease the angiogenic response is typically robust, though it can be impaired in conditions such as hypercholesterolemia and diabetes where the endothelium is dysfunctional. Myoglobin (Mb) is expressed exclusively in striated muscle cells. We hypothesized that myocyte specific overexpression of myoglobin attenuates ischemia-induced angiogenesis even in the presence of normal endothelium.

**Methods and Results**—Mb overexpressing transgenic (MbTg, n=59) and wild-type (WT, n=56) C57Bl/6 mice underwent unilateral femoral artery ligation/excision. Perfusion recovery was monitored using Laser Doppler. Ischemia-induced changes in muscle were assessed by protein and immunohistochemistry assays. Nitrite/nitrate and protein-bound NO, and vasoreactivity was measured. Vasoreactivity was similar between MbTg and WT. In ischemic muscle, at d14 postligation, MbTg increased VEGF-A, and activated eNOS the same as WT mice but nitrate/nitrite were reduced whereas protein-bound NO was higher. MbTg had attenuated perfusion recovery at d21 (0.37±0.03 versus 0.47±0.02, P<0.05), d28 (0.40±0.03 versus 0.50±0.04, P<0.05), greater limb necrosis (65.2% versus 15%, P<0.001), a lower capillary density, and greater apoptosis versus WT.

**Conclusion**—Increased Mb expression in myocytes attenuates angiogenesis after hind-limb ischemia by binding NO and reducing its bioavailability. Myoglobin can modulate the angiogenic response to ischemia even in the setting of normal endothelium. (Arterioscler Thromb Vasc Biol. 2008;28:2144-2150.)

**Key Words:** angiogenesis ■ animal models of human disease ■ genetically altered mice ■ endothelium/vascular type/nitric oxide

Angiogenesis, the process of formation of new blood vessels, can be therapeutic, as in endogenous response to arterial occlusions or when one seeks to deliver growth factors to increase blood flow and treat disorders of inadequate tissue perfusion.1–3 Nitric oxide (NO) plays a crucial role in both the endogenous angiogenic response to ischemia and in the “therapeutic response” sought after administration of growth factors.4–6 It has been established that imbalance in NO production or availability leads to impaired angiogenesis. The bioavailability of NO in tissue depends on its production and stability. Most studies focusing on NO deregements in tissue have used models of severe endothelial dysfunction, where there is reduced NO production attributable to dysfunctional endothelial NO synthase (eNOS),9 or reduced half-life of NO attributable to oxidative stress,9,10

Recent evidences indicate that factors outside the endothelium can also regulate NO bioavailability.11,12 Myoglobin is a protein of oxidative metabolism expressed almost exclusively in myocytes of cardiac and striated skeletal muscle, and has been shown to interact with NO.11 Human myoglobin has been shown to react with NO to yield heme-NO (ferrous nitrosyl-Mb) in the absence of dioxygen and to form S-nitrosylated myoglobin (SNO-Mb) in presence or absence of oxygen.13 The interactions of myoglobin with NO can result in scavenging NO11,14 or in yielding more biologically stable NO at sites away from the site of synthesis.15,16 Hearts from Mb−/− mice showed an increased sensitivity (vasodila-tation and cardiodepression) in response to NO and bradyki-nin, indicating a negative role of myoglobin in NO availability.11 On the contrary, deoxymyoglobin can generate NO by reduction of nitrite and thereby regulate mitochondrial respiration17 and can be cytoprotective during ischemia-reperfusion by decreasing cellular energy consumption.18 Thus, data from published literature on NO-Mb interaction could be predictive of a beneficial or an adverse outcome on NO-dependent pathways. This study was designed to test the hypothesis that manipulating myoglobin expression in myocytes can alter NO availability and thus modulate angiogenesis after hind-limb ischemia in the setting of normal endothelial function.
Materials and Methods

Experiment Design and Groups

In total, 56 wild-type (WT) and 59 myoglobin transgenic (MbTg) mice were studied. Ten mice per group were used for measurement of protein expressions and capillary density at baseline. Twenty mice per group were used for measurement of perfusion recovery after hind-limb ischemia and followed for 28 days. After 28 days, the gastrocnemius muscle was harvested and used for assessment of capillary density and apoptosis. Another 20 mice from each group were euthanized at 14 days after hind-limb ischemia for protein expressions (n=10 per group) or NO measurements (n=10 per group). Aortas from a separate subset of mice (n=6 per group) were used for assessment of vascular reactivity. Animal study protocols were approved by Duke University’s Institutional Animal Care and Use Committee.

Myoglobin Transgenic Mice

Myoglobin-overexpressing transgenic mice in an ICR background were obtained from the laboratory of R. Sanders Williams, MD (Duke University) and were then backcrossed 9 generations into C57Bl/6 mice. The transgene contains the intact myoglobin gene including the promoter regions, so that it is expressed only in cardiac and skeletal muscle, as shown previously. The ninth generation C57Bl/6 MbTg mice and WT litter mates were used for the study. MbTg mice were identified using markers previously described.

Surgical Induction of Hind-Limb Ischemia and Hemodynamic Assessment

Unilateral hind-limb ischemia was induced and hind-limb perfusion was measured as described previously. At the indicated time points, postligation perfusion was assessed and the data were expressed as the ratio of perfusion in the ischemic limb to that in the contralateral nonischemic limb. The extent of limb necrosis was scored using the following convention: Stage I: necrosis of toes; Stage II: necrosis extending to dorum pedis; Stage III: extending to crus; and stage IV: Extending to thigh or complete limb. Mice with grade II necrosis and above (3/23 mice from the MbTg group) were excluded from the perfusion analysis.

Protein Analysis, Measures of Capillary Density and Apoptosis

Statistics

Measurement of Nitric Oxide Synthase Activity and Vascular Reactivity

NOS activity was measured in frozen sections from ischemic muscle while both NOS and vasoreactivity were measured in aortas. See online supplement of Materials and Methods.

Assessment of Skeletal Muscle Atrophic Response to Endotoxin Injury

To determine whether skeletal muscle from MbTg mice is more susceptible to direct injury than WT, we determined the muscle atrophy response of mice after injection of endotoxin, as described. Twenty-four hours after the endotoxin injection, mice were euthanized and mRNA expression of MAfBx/Atrogin-1 was quantified in gastrocnemius muscle using Taqman Assay for Atrogin-1 (Applied Biosystems). Endogenous 18s was used for normalization and comparison of mRNA copy number was done using comparative ΔCt method.

Results

MbTg Versus Wild Type: Skeletal Muscle Vasculature and Aortic Vasoreactivity

Protein expression of transgene in gastrocnemius muscle of MbTg mice was identified by immunoblotting (Figure 1A) because the HA-tagged myoglobin (HA-Mb) migrates slower in SDS-PAGE than the endogenous myoglobin protein (Mb). Total muscle myoglobin was approximately 2-fold higher (P<0.001) by ELISA in the transgenic compared to WT mice (Figure 1B). Skeletal muscle from MbTg mice had significantly higher expression of VEGF-A compared to WT mice (Figure 1C). However, capillary density (Figure 1D) and the expression of total eNOS and activated total (phosphorylated/total) eNOS (p-eNOS/eNOS) were similar between the groups (Figure 1F). The contractile responses of the isolated aortic rings to 100 nmol/L K+ and phenylephrine (PE) were
comparable between the two groups (maximal active stress normalized to cross sectional area of ring: 100 mmol/L KPSS, WT versus MbTg 5.2±0.8 versus 6.7±2.5 mg/mm² and 30 μmol/L PE, WT versus MbTg 6.7±1.4 versus 5.5±1.3 mg/mm², both P=NS). Also, ACh and SNP induced relaxation responses showed that the ED₅₀ for ACh and SNP were comparable between the groups (Figure 1F). NOS activity in aorta, as detected in frozen sections and cGMP levels in homogenates (supplemental Figure IA and IB) were not different between the groups. As expected, vascular smooth muscle did not stain positive for myoglobin (cross section of aorta shown in supplemental Figure II), thus confirming that the transgene was not ectopically expressed in vascular smooth muscle.¹⁹ These findings indicate that MbTg and WT mice have similar (normal) endothelial function and have no differences in NOS activity and levels of NO within the vasculature.

MbTg Versus Wild-Type: Attenuated Perfusion Recovery, Reduced Capillary Density, Increased Apoptosis and Increased Necrosis After Hind-Limb Ischemia

Immediately after induction of hind-limb ischemia, there was no difference in the perfusion ratio between the two groups (d0, perfusion ischemic/nonischemic limb; WT versus MbTg 0.30±0.01 versus 0.29±0.01; P=NS). The extent of perfusion recovery between the MbTg and WT groups was not significantly different at 7-, 10-, and 14-day postligation time points. However, the perfusion ratio was significantly lower in MbTg versus WT at 21 (0.65±0.02 versus 0.75±0.03, P<0.05) and 28 (0.68±0.03 versus 0.78±0.03, P<0.05) days after ligation (Figure 2A). In the 28-day posts ischemic gastrocnemius muscle, capillary density was significantly less in MbTg mice compared to WT (Figure 2B, WT 0.31±0.04 versus MbTg 0.18±0.03 capillaries/fiber; n=8; P<0.05). There was no difference in macrophage infiltration or number of smooth muscle positive blood vessels in the ischemic muscle between the two groups (supplemental Figure IIIA and IIIB).

There was increased apoptosis in the ischemic muscle of MbTg compared to that in WT as determined by caspase-3 activity (Figure 2C) and tunel staining (Figure 2D). In addition, the incidence of limb necrosis was significantly higher in MbTg (65.2%) compared to wild-type (15%) and when present, the degree of necrosis was greater in MbTg mice (Table).

Both MbTg and Wild Type Increase VEGF-A Expression, Activate eNOS, and Generate NO to Similar Degrees

As shown in Figure 1, differences in perfusion recovery between groups could not be accounted for by findings in the muscle preligation. Indeed, the higher levels of VEGF-A in nonischemic muscle in the MbTg mice (Figure 1B) might have been expected to improve perfusion recovery. We next sought to determine whether the “angiogenic” response to ischemia was similar between the transgenic and wild-type mice. VEGF-A and the ratio of p-eNOS/total eNOS was similar between MbTg and WT mice at the time point (14-day) when perfusion recovery was similar between the groups. In both groups, there was a comparable increase in VEGF-A in the ischemic compared to the nonischemic limb (Figure 3A). After ischemia, both MbTg and WT mice had upregulation of p-eNOS (Figure 3B). There was no difference between the groups. In addition, in 14-day posts ischemic tissue, there was no difference in total NOS activity between MbTg and WT mice (Figure 3C), thereby cumulatively indicating that both groups had comparable production of NO.

MbTg Versus Wild Type Postischemia: Reduced Tissue Levels of Total NO With Higher Protein-Bound NO

We measured the levels of nitrite and nitrate in skeletal muscle as indicators of NO in the tissue 14 days after ischemia, when perfusion recovery in both groups was comparable. Total NO in the ischemic limb was significantly less in the MbTg mice compared to that in WT (Figure 4A,
WT 34.2±1.9 versus MbTg 29.9±0.8 μmol/L/mg protein; n=10; *P<0.05). We determined the levels of nitrate and nitrite in the rodent chow and water. Whereas regular laboratory water had nitrate/nitrite levels of 63.7±0.2 μmol/L/mL, these levels were undetectable in the water used in the animal facilities. The rodent chow had nitrate/nitrite levels of only 6.4±5 μmol/L/mg. Because both groups received the same diet and water, dietary source should not account for the difference in tissue nitrate/nitrite. We also measured the tissue levels of cGMP as an alternative measure of tissue NO. Whereas cGMP levels were similar between the groups in the nonischemic limb, ischemic tissue from MbTg mice had

Table. Incidence and Grades of Limb Necrosis in WT vs MbTg Mice After Hind-Limb Ischemia

<table>
<thead>
<tr>
<th>Grades of necrosis</th>
<th>Total No.</th>
<th>No. of Necrosis</th>
<th>Incidence of Necrosis</th>
<th>0</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>20</td>
<td>3</td>
<td>15%</td>
<td>17</td>
<td>3 (15%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MbTg</td>
<td>23</td>
<td>15</td>
<td>65.2%*</td>
<td>8</td>
<td>12 (52%)</td>
<td>2 (9%)</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
</tbody>
</table>

*P<0.001 vs WT.

Figure 2. A, Time course (left) and image (right) of laser Doppler perfusion imaging (LDPI) shows myoglobin-transgenic (MbTx) had impaired perfusion recovery compared to wild-type (WT) mice, day 21 and 28 after hind-limb ischemia (n=20 per group, *P<0.05). B, Ischemic muscle from MbTg mice (1, 28 days after ischemia) had lower capillary density compared to WT, indicating an attenuated angiogenic response (n=8 per group, *P<0.05) with more apoptosis by (C) caspase-3 activity (n=8 per group, *P<0.01) and (D) Tunel staining (n=8 per group, *P<0.05).
significantly lower levels of cGMP (Figure 4B, 5.0±0.8 versus 8.2±1.0 pM/mg protein in WT, n=8, P<0.05). The levels of protein-bound NO were significantly higher in muscles from MbTg mice compared to that from WT (Figure 4C, WT 2.8±0.6 versus MbTg 8.8±3.1 nmol/L/mg protein; n=10; n=9; P<0.05). There was no difference in the values following pretreatment of the samples with mercuric chloride, thereby indicating that the protein-bound NO existed predominantly in the ferrous-nitrosyl-Mb form.

Lack of Increased Muscle Injury in MbTg Versus WT With Endotoxin Injury

The expression of Atrogin-1 mRNA (Atrogin/18s copy #, mean±SEM) was not different between the groups (MbTg 0.39±0.2 versus WT 0.56±0.3, n=4/group, P=NS) after endotoxin induced muscle injury.

Discussion

The results from this study demonstrate, for the first time, that processes within myocytes can directly modulate angiogenesis in the setting of normal endothelial function. Our data add to a well established literature that NO plays an important role in angiogenesis, but these other studies have largely focused on the endothelium as the site for altered NO homeostasis. Myoglobin is a muscle-specific protein that is present only in striated skeletal muscle. Accumulating evidence indicates that NO interacts with myoglobin to yield different products, but the definitive functional role of myoglobin-NO interactions remains to be elucidated.

Using a transgenic model, we show the first evidence that myoglobin can play a significant role in modulating the endogenous angiogenic response to ischemia by reducing NO bioavailability, in part via sequestering NO as nitrosyl-heme.
The impaired angiogenic response occurred despite increased levels of VEGF-A, and activation of eNOS after ischemia. Data from this study that perfusion recovery in the MbTg mice is significantly attenuated only at late time points (day 21 and 28) after ischemia is consistent with an impaired angiogenic response, as opposed to differences in preexisting collaterals. We could find no difference in macrophage infiltration between the ischemic tissues of MbTg vs wt mice (supplemental Figure III). The aortic ring studies, where myoglobin is not locally expressed, confirm that MbTg mice have similar endothelial function, NOS activity and NO availability in the vasculature, as wild-type mice.

Myoglobin has traditionally been characterized as the oxygen carrier that is abundantly expressed in the skeletal muscle. The results of our study clearly support the work of others to suggest that myoglobin also plays a vital role in NO-mediated signaling pathways in angiogenesis, but to our knowledge, there is no evidence that myoglobin has a direct role in NO-mediated signaling pathways in angiogenesis, but this cannot be ruled out completely. Despite the implications of myoglobin–NO interactions being cytotoxic and impairing angiogenesis and perfusion recovery.

In summary, data from this study show that increased myoglobin expression in skeletal myocytes attenuates perfusion recovery after hind-limb ischemia, in part, by scavenging NO and reducing its bioavailability. This study provides the first direct evidence that myoglobin levels can serve as significant regulators of bioavailable NO in skeletal muscle and can modulate the angiogenic response in an endothelium independent manner. In addition, this describes a novel model for impaired ischemic angiogenesis for studying NO biology.

Acknowledgments
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Figure 4. A, The sum of stable NO products nitrate and nitrite was measured as an index of the available NO. At 14 days postoperatively, ischemic skeletal muscle from myoglobin-transgenic mice (MbTg) had significantly less NO compared to wild-type (WT) mice (n=10 per group; *P<0.05). B, cGMP levels did not differ in the nonischemic limb, but were significantly lower in the ischemic muscle from MbTg vs wt mice (n=8 per group, *P<0.05). C, MbTg muscle had significantly higher levels of protein-bound NO compared to WT mice; indicating NO scavenging by myoglobin (n=9 per group; *P<0.05).
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Disclosures
None.

References
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 appropriate 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 (ED₅₀) 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.
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).