Endothelial-Specific Expression of Mitochondrial Thioredoxin Promotes Ischemia-Mediated Arteriogenesis and Angiogenesis

Shengchuan Dai, Yun He, Haifeng Zhang, Luyang Yu, Ting Wan, Zhe Xu, Dennis Jones, Hong Chen, Wang Min

**Objective**—Thioredoxin-2 (Trx2), a major antioxidant protein in mitochondria, enhances nitric oxide bioavailability and inhibits ASK1-dependent apoptosis in endothelial cells (ECs). However, the in vivo role of Trx2 in angiogenesis has not been defined. Here we used EC-specific transgenesis of Trx2 (Trx2-TG) in mice to determine the in vivo function of Trx2 in arteriogenesis and angiogenesis.

**Methods and Results**—In a femoral artery ligation model, Trx2-TG mice had enhanced capacity in limb perfusion recovery and ischemic reserve capacity compared to the nontransgenic littermates. Ischemia-initiated arteriogenesis in the upper limb was augmented in Trx2-TG mice. Trx2-TG mice also showed significantly enhanced capillary formation and maturation in the lower limb. In nontransgenic limb, ischemia specifically induced a downregulation of Trx2 protein, leading to increased oxidative stress, ASK1 activation, and EC apoptosis. In contrast, Trx2-TG maintained a constitutive level of Trx2, reducing the ischemia-induced deleterious responses. We then defined the mechanism by which Trx2 increases angiogenesis using ECs isolated from Trx2-TG mice. Trx2-TG ECs showed increased NO and NO-dependent migration. In addition, these cells were more resistant to oxidative stress–induced activation of ASK1 signaling and apoptosis. Moreover, Trx2-augmented NO and Trx2-reduced ASK1 apoptotic activity to angiogenesis in vivo, we examined Trx2 effects on ischemia-induced angiogenesis in eNOS-deficient mice. The eNOS deletion caused severe impairment in the functional flow recovery in response to ischemia. Trx2 expression in eNOS-KO mice still dramatically inhibited ischemia-induced ASK1 and EC apoptosis, leading to an enhanced functional flow recovery.

**Conclusion**—These in vivo and in vitro data support that Trx2 maintains EC function by two parallel pathways—scavenging ROS to increase NO bioavailability and inhibiting ASK1 activity to enhance EC survival, facilitating ischemia-mediated arteriogenesis and angiogenesis. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

**Key Words:** thioredoxin ■ angiogenesis ■ ischemia ■ apoptosis

**Angiogenesis** is a process of new blood vessel formation, which contributes to various physiological processes and pathological settings. While excessive angiogenesis links atherosclerosis, cancer, and diabetic retinopathy, defects in angiogenesis directly contribute to myocardial infarction and peripheral arterial disease. Recent reports suggest that reactive oxygen species (ROS) can positively or negatively regulate angiogenesis. Although physiological levels of ROS are required for angiogenesis, excess amount of ROS generated during inflammation and ischemic response may inhibit reparative vascular remodeling by inducing endothelial dysfunction and apoptosis. ROS-producing systems in vascular endothelial cells (ECs) are numerous including various NADPH oxidases, xanthine oxidase, the uncoupling of NO synthase as well as mitochondria. The NADPH oxidases have been considered predominant sources of ROS in the pathogenesis of cardiovascular diseases. Recent data support that ROS generated from mitochondria significantly regulate EC function. Physiological ROS may function as a second messenger in signal transduction and regulate EC growth/proliferation, apoptosis, EC barrier function, vasorelaxation, and vascular remodeling. However, cardiovascular risk factors often induce mitochondria dysfunction, leading to overproduction of ROS. These excess ROS induce EC dysfunction by reducing NO bioavailability and activating apoptotic signaling.

The role of ROS as signaling molecules in growth factor–mediated angiogenesis has been investigated. One of the
major sources of ROS in ECs is NADPH oxidase which consists of Nox family proteins, p22phox, p47phox, p67phox, and the small G protein Rac1. In vitro and in vivo function of NADPH oxidase in angiogenesis has been defined in several mouse models. Neovascularization in an ischemic hindlimb model is significantly impaired in gp91phox-KO mice, likely because of reduction of superoxide produced from inflammatory cells as well as neovascularure. On the contrary, negative regulation of angiogenesis by ROS has also been reported from studies with ROS-scavenging enzymes. Mice lacking ecSOD (ecSOD-KO) have impaired ischemia-induced increase in collateral vessel formation, capillary density, and blood flow recovery. This impairment correlates with enhanced superoxide production, decreased NO activity, and increased apoptotic cells in ecSOD-KO mice. Thus, angiogenesis is delicately balanced by both these cytosolic ROS-generating oxidases and ROS-scavenging enzymes.

However, the roles of mitochondria-derived ROS and mitochondrial antioxidant system in angiogenesis have not been addressed. The mitochondrial thioredoxin (Trx2) system appears to be a critical component to maintain mitochondrial normal function. Trx2-dependent peroxidase (Prx3) and reductase (TrxR2), together with MnSOD (SOD2), provide a primary line of defense against ROS produced by the mitochondrial respiratory chain. In support of this notion, mice with a deletion of Trx2 or TrxR2 display embryonic lethality, likely because of increased oxidative stress. Similarly, cells with deficiency or knockdown of Trx2 or Prx3 accumulate endogenous ROS and are highly sensitive to exogenous oxygen radicals. However, little is known about the function of the Trx2 system in the vasculature.

Previously we have shown that EC-specific transgenesis of Trx2 (Trx2-TG) improves aortic EC function and reduces atherosclerotic lesions at aortic roots in the Apo E-deficiency mouse model by reducing oxidative stress. However, the role of Trx2 in microvessels has not been investigated. In the present study, we demonstrate a critical role of Trx2 in ischemia-mediated reparative arteriogenesis and angiogenesis in a femoral artery ligation model using the aforementioned Trx2-TG mice.

**Methods**

The detailed materials and methods are provided in the online supplement (available online at http://atvb.ahajournals.org). Most of the methods have been previously published, including generation of the EC-specific Trx2 transgenic mice, mouse hindlimb ischemic model, postcontraction hyperemia, histology and immunohistochemistry, gene expression in ischemic muscle, cell culture and cytokines, EC migration assay and image analysis, immuno-blotting, ASK1 and JNK kinase assays, and quantification of apoptosis.

**Results**

**Trx2-TG Augments Perfusion Recovery in Ischemic Hindlimbs**

Nontransgenic littermates (WT) and Trx2-TG male mice were subjected to femoral artery ligation. Blood flow in ischemic and nonischemic limb perfusion was measured presurgery and postsurgery as indicated (days 0 [30 minutes], 3, 7, and 14). Trx2-TG mice were higher in baseline blood flow (supplemental Figure 1), correlating with a lower aortic pressure in Trx2-TG compared to WT mice as we observed previously. Before surgery, the ratio of left leg to right leg gastrocnemius blood flow was 1. Thirty minutes postsurgery, flow dropped to 0.2 and returned to a ratio of 0.8 over 2 weeks in WT mice. Trx2-TG mice showed augmented recovery of hindlimb perfusion on days 3 and 7 and the limb flow returned to normal on day 14 (Figure 1a). To further define the functional defects in mice, we examined skeletal

![Figure 1](https://via.placeholder.com/150)

Figure 1. Critical roles of Trx2 in the recovery of hindlimb perfusion postinjury. a, Trx2-TG mice show augmented recovery of limb perfusion compared to nontransgenic WT mice. Ischemic hindlimbs were performed, and blood flow of ischemic and nonischemic limb were measured on gastrocnemius muscle at 30 minutes, 3, 7, and 14 days after surgery. Ratio of perfusion unit from nonischemic (left) to ischemic limbs (right) are shown. n indicates number for each strain is shown in parenthesis.
muscle contraction stimulated hyperemia in the gastrocnemius muscle in WT and Trx2-TG mice at baseline and after ischemia as we described recently for Bmx-KO and TNFRII-KO mice.20,21 As seen in the representative traces in Figure 1b, electric stimulation of the adductor muscle groups in the upper leg of WT and Trx2-TG mice results in a marked increase in peak blood flow [compare prestimulation (Pre-Sti) to the peak response of stimulation (time 0)]. Trx2-TG mice had greater blood flow compared to WT and a delayed return to baseline compared to WT mice, suggesting that Trx2-TG mice have reduced peripheral vascular resistance. These data are consistent with the high NO activity in Trx2-TG mice as NO is critical for maintaining vasodilation necessary for the gradual return of flow back to normal.19

Next, we measured the same physiological response after limb ischemia in WT and Trx2-TG mice at 2 weeks posts ischemia. Electric stimulation of the adductor muscle groups in the upper leg of WT mice after 2 weeks surgery showed only 70% of the peak response measured in the gastrocnemius muscle group compared to preischemia. However, Trx2-TG mice had a complete recovery in peak blood flow (Figure 1b preischemia versus Figure 1c postischemia, 105 ± 5%). These data show that Trx2-TG mice have augmented perfusion recovery in ischemic hindlimbs.

**Postischemic Arteriogenesis and Angiogenesis Are Enhanced in Trx2-TG Mice**

Enhanced limb perfusion could be attributable to increased arteriogenesis from existing vessels of the upper limb and increased neovascularization/vessel maturation in the lower limb. Previously we have visualized vascular architecture by Microfil casting to determine ischemia-initiated collateral artery growth (arteriogenesis).20,21 We compared the collateral growth in WT and Trx2-TG mice by the same approach. There was an enlargement of collaterals compared to contralaterals in WT mice after 2 weeks of ischemia. Trx2-TG showed enhanced arteriализation as determined by the ratio of vascular density in the left arteries versus the right arteries (supplemental Figure II). Interestingly, the Trx2-TG mice showed greatly enhanced vessel sprouting, consistent with the increased NO activity in these mice19 which has been shown to mediate vessel branching and morphogenesis.24 To determine whether neovascularization and vessel maturation in the lower limb are increased in Trx2-TG mice, we performed immunohistochemistry with EC- and pericyte-specific markers. After 7 and 14 days of ischemia, there was an increase in CD31-positive capillaries surrounding the skeletal muscle fibers in WT mice (Figure 2a for day 7 with quantification of the number of capillaries and capillary/fiber ratio in Figure 2b and 2c, respectively). Trx2 expression did not alter the cross-section of muscle fibers and muscle morphology (Figure 2d). However, Trx2-TG mice show increased numbers of dilated vessels as well as SMA-positive capillaries, consistent with the role of NO in vessel stabilization.24 Ischemic-induced vessel maturation as determined by smooth muscle α-actin (SMA) staining was also increased (supplemental Figure II for day 14 with quantification of SMA-positive capillaries/mm²). Consistent with the results that Trx2-TG mice showed much greater recovery in hind-limb perfusion compared to WT mice, CD31-positive capillaries surrounding the skeletal muscle fibers (neovascularization) and SMA-positive SMC (pericyte recruitment) were significantly increased in Trx2-TG mice compared to WT secondary to ischemia on both days 7 and 14 (Figure 2 and supplemental Figure II).

**Trx2 Reduces Oxidative Response, ASK1-JNK Activation, and Cellular Apoptosis in Ischemic Tissue**

To understand how Trx2-TG promotes angiogenesis, we first measured Trx2 mRNA (supplemental Figure III) and protein expression (supplemental Figure III) in response to ischemia. We found that endogenous Trx2, but not other antioxidant proteins Trx1, SOD1 or SOD2, was drastically reduced in nontransgenic mice in response to ischemia (supplemental Figure III). In contrast, Trx2 transgene was resistant from ischemia-induced downregulation. We measured ischemia-induced ROS production and activation of ASK1-JNK signaling and infiltration of leukocytes. Oxidative stress in muscle tissue was determined by an in situ detection of superoxide with dihydroethidium fluorescence (DHE) and by immunostaining with antibody against nitrotyrosine, an indicator of peroxynitrite-induced tyrosine nitrosylation. DHE intercalates into DNA and gives fluorescence primarily from the nucleus. Ischemia on day 7 strongly induced production of superoxide, apparently from vascular ECs and infiltrated cells but not from myocytes (Figure 3a). However, basal and ischemia-induced oxidative stress were significantly reduced in Trx2-TG mice (Figure 3a and 3b). Similar results were obtained for nitrotyrosine staining (supplemental Figure IV). Inflammatory response was examined by leukocyte infiltration with anti-CD45 antibody, and Trx2-TG mice showed significantly reduction in leukocyte infiltration compared to WT mice (supplemental Figure IV). The reduction of leukocyte infiltration is consistent with the increased NO activity in Trx2-TG mice. It is also correlated with reduced ROS production and oxidative stress in Trx2-TG mice. ASK1-JNK/p38 MAPK signaling pathway can be activated by oxidative stress and inflammatory stimuli.25 Activation of ASK1-JNK/p38 was determined by Western blot with phospho-specific antibodies, and was strongly induced in ischemia hindlimb on day 7. However, Trx2 significantly blunted ischemia-induced activation of ASK1-JNK/p38 signaling (Figure 3c). We then measured tissue apoptosis by TUNEL assay and cellular proliferation by PCNA staining. Kinetics studies indicated that apoptosis peaked at day 7 postsurgery, suggesting that ischemia-induced apoptosis is an early event in the adaptive response.21 More importantly, ischemia-induced apoptosis was dramatically decreased in Trx2-TG mice compared to WT mice (supplemental Figure IV). Cellular proliferation started at day 7 and sustained until 4 weeks (not shown, also see25). Both capillaries and myocytes showed PCNA-positive staining (supplemental Figure IV, arrowheads and arrows, respectively). These data suggest that Trx2-TG reduces oxidative stress, leading to reduced ASK1-JNK/p38 signaling and apoptosis while yet enhancing cellular proliferation in ischemic tissue.
To determine whether the in vivo function of Trx2 in angiogenesis correlates to in vitro activities of Trx2 in EC, we examined the effects of Trx2 on EC migration and survival which are critical components of the angiogenesis process. Mouse microvessel ECs (MECs) from lung were isolated from WT and Trx2-TG mice, and the effect of Trx2 on VEGF-induced EC migration was first determined in a monolayer injury model. Trx2-TG MECs showed increased migration compared to WT MECs (Figure 4a and supplemental Figure V). Consistent with our previous finding, Trx2 augments NO bioavailability in MECs by measuring nitrite release into the culture media using an NO-specific chemiluminescence (Figure 4b) without effects on eNOS phosphorylation. Addition of an eNOS inhibitor L-nitro arginine methyl ester (L-NAME, 100 μmol/L) blocked NO release (Figure 4b) as well as EC migration in both WT and Trx2-TG cells (Figure 4a), suggesting that NO contributes to Trx2-augmented EC migration. To further Trx2 in angiogenic signaling, Trx2 expression was suppressed by Trx2 siRNA as we described previously. Trx2 siRNA, but not a control siRNA, knocked down Trx2 expression by 90%. However, Trx2 knockdown had no effects on expression of Trx1 (Figure 4c) or other antioxidant proteins (SOD1 and SOD2, not shown). Trx2 knockdown significantly reduced NO bioavailability (Figure 4c) and EC migration under a normal EC culture condition (Figure 4d). To determine the effects of Trx2 on EC survival, WT and Trx2-TG MECs were treated with TNF (10 ng/mL) in the presence of cycloheximide (CHX, 10 μg/mL). TNF is known to induce superoxide in ECs and mitochondria-dependent apoptosis. Consistent with our previous findings that Trx2 protects ECs from apoptosis by inhibiting ASK1 activity, TNF-induced ASK1 activity was significantly reduced in Trx2-TG MECs (Figure 4e). EC apoptosis was determined by FACS analysis with FITC-conjugated annexin V or DAPI staining for nuclear fragmentation. Trx2-TG MECs showed dramatically reduced apoptosis by annexin V-staining (Figure 4f and supplemental Figure V) and nuclear fragmentation assay (not shown). A similar inhibition of Trx2 on H₂O₂-induced EC death was observed (not shown).

**Both NO and ASK1 Pathways Contribute to Ischemia-Induced Angiogenesis**

To define the relative contributions of Trx2-increased NO and Trx2-reduced ASK1 apoptotic activity to angiogenesis in...
vivo, we examined Trx2 effects on ischemia-induced angiogenesis in eNOS-deficient mice. The eNOS-KO/Trx2-TG mice were obtained by mating with Trx2-TG with eNOS-KO mice (both eNOS-KO and Trx2-TG mice are C57Bl/6 background; supplemental Figure VI) and Trx2 protein expression in Trx2-TG mice was not altered after crossing with eNOS-KO mice (see Figure 5b). We have added the blot into the revised Figure 5b. eNOS-KO and eNOS-KO/Trx2-TG mice were subjected to femoral artery ligation, and activation of ASK1, functional blood flow, and morphological analysis were performed as described for WT and Trx2-TG mice. A deletion of NOS had no significant effects on ASK1 activity (Figure 5a). Trx2 expression in eNOS-KO mice significantly inhibited ischemia-induced ASK1 apoptotic signaling (Figure 5b). Consistent with previous findings,26 the eNOS deletion caused severe impairment in functional flow recovery in response to ischemia (Figure 5c). Similar to the results obtained from Trx2-TG (Figure 2), Trx2 expression in eNOS-KO mice also led to increased angiogenesis, numbers of dilated vessels (supplemental Figure VII) and flow recovery (Figure 5c). However, ischemia-induced angiogenesis and functional flow in eNOS-KO/Trx2-TG mice were still less than those observed in WT and Trx2-TG mice. These data strongly support that both Trx2-increased NO and Trx2-reduced ASK1 apoptotic activity contribute to in vivo angiogenesis.

**Discussion**

The most important finding of this study is that mitochondrial antioxidant protein Trx2 plays a critical role in ischemia-mediated arteriogenesis and angiogenesis, leading to an enhanced tissue reparative response. Specifically, EC-specific transgenesis of Trx2 enhances capacity in limb perfusion and ischemic reserve capacity compared to the nontransgenic wild-type mice. Immunohistochemical analyses indicate that Trx2-TG mice had increased ischemia-initiated EC proliferation, neovascularization, and vessel maturation. In nontransgenic mice, the Trx2 protein (but not other antioxidant proteins such as Trx1, SOD1, or SOD2), is dramatically downregulated in response to ischemia. The reduction of Trx2 protein correlates with concomitant increases in oxidative stress, leukocyte infiltration, ASK1 activation, and cellular apoptosis. Interestingly, EC-specific Trx2 transgenesis renders a constitutive level of Trx2 with blunted ischemia-induced oxidative responses. In vitro studies suggest that ECs isolated from Trx2-TG mice show...
increased NO bioavailability and NO-dependent EC migration. These cells are also more resistant to oxidative stress–induced ASK1 signaling and apoptosis in an NO-independent manner. We further express Trx2 in eNOS-deficient mice to demonstrate that both Trx2-increased NO and Trx2-reduced ASK1 apoptotic activity contribute to in vivo angiogenesis. (Figure 5d, proposed model for Trx2 function). Our study provides the first evidence that a mitochondrial antioxidant protein plays a critical role in vascular remodeling.

Genetic deletion of Trx1, Trx2, or TrxR2 causes embryonic lethality attributable to overproduction of ROS,16,17,27 suggesting that thioredoxin system is the first defense mechanism against ROS. Therefore, we took a Trx2-transgenesis approach and demonstrate that Trx2-TG specifically ex- 

Antiapoptotic activity is another important function of Trx2. We have previously demonstrated a critical role of ASK1 in TNF/ROS-induced mitochondria-dependent apoptotic signaling pathway. We showed that ASK1 is localized in the cytosol as well as in mitochondria. Trx2, like Trx1,29 directly binds to ASK1, and forms a complex with ASK1 in mitochondria, inhibiting ASK1 activity.23 Angiogenic factors such as bFGF can induce a translocation of survival protein Raf-1 to EC mitochondria where it also associates with and


directly binds to ASK1, and forms a complex with ASK1 in mitochondria, inhibiting ASK1 activity.23 Angiogenic factors such as bFGF can induce a translocation of survival protein Raf-1 to EC mitochondria where it also associates with and
increased ASK1 activation and apoptotic injury in hindlimb. In contrast, Trx2-TG mice show resistance to ischemia-induced downregulation of Trx2 resulting in increased ASK1 activation and apoptotic injury in hindlimb. Similarly, ischemia-induced downregulation of Trx2 in ECs does not eliminate effects of Trx2 inhibition on ASK1 and tissue apoptosis in vivo. It has been shown that NO contributes to antiapoptosis, in part, by directly nitrosylating and inhibiting proapoptotic proteins including caspases, ASK1, and Trx1. Although it is not known whether ASK1 is modified by NO from eNOS in ECs, our data suggest that NO does not significantly contribute to Trx2-enhanced antiapoptotic activity in ECs.

The mechanism for Trx2 downregulation by ischemia is not clear, although the regulation of Trx1 by ROS has been extensively investigated. It is shown that low doses of reactive oxygen species protect ECs from apoptosis by increasing Trx1 expression. This upregulation appears to be at the transcriptional level. However, high levels of ROS can directly induce Trx1 protein degradation in a catabsin-dependent manner. Cathepsin D is a lysosomal aspartic proteinase and plays an important role in the degradation of proteins and in apoptotic processes induced by oxidative stress and cytokines. Our results indicate that Trx2 is regulated by ROS in a different manner as Trx1. This is supported by our data that Trx2 but not Trx1 is downregulated by ischemia. Ischemia appears to regulate Trx2 at a transcriptional level. Further studies are required to define the mechanism for the Trx2 regulation in pathological settings. In conclusion, our data strongly support that Trx2, by reducing oxidative stress, enhancing NO bioactivity, and improving EC survival, plays a critical role in ischemia-mediated arteriogenesis and angiogenesis. Trx2 may be a novel target for the treatment of vascular diseases such as coronary artery disease and peripheral arterial disease.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIALS

Endothelial-specific expression of mitochondrial thioredoxin promotes ischemia-mediated arteriogenesis and angiogenesis

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MATERIALS AND METHODS

Animal protocol. Mice were housed in specific pathogen-free animal facilities according to guidelines from the Office of Laboratory Animal Welfare at the National Institute of Health, and all experimental procedures were approved by the institutional animal care and use committees at Yale University.

Generation of the EC-specific Trx2 transgenic mice. Generation of Trx2 transgenic mice (Trx2-TG) in which Trx2 gene is driven by an VE-cadherin promoter has been described previously 1. TG mice were backcrossed with C57BL/6 mice for more than 10 generations. All experiments were performed with heterozygous Trx2-TG mice and their non-TG littermates as controls. The eNOS-KO/Trx2-TG mice were obtained by mating with Trx2-TG with eNOS-KO mice (in C57Bl/6 background from William Sessa’s lab at Yale).

Mouse hindlimb ischemic model. 8–12 week old male non-TG (WT) and Trx2-TG mice were used for all experiments. Mouse ischemic hindlimb model was performed as described previously 2. Briefly, following anesthesia (79.5 mg/kg ketamine, 9.1 mg/kg; xylazine), the left femoral artery was exposed under a dissection microscope. The proximal of femoral artery and the distal portion of saphenous artery were ligated. All branches between these two sites were ligated or cauterized, and arteriectomy was performed. Sham operation is without femoral artery ligation but skin incision. Blood flow was measured by PeriFlux system with Laser Doppler Perfusion Module (LDPU) unit (Perimed, Inc. North Royalton, OH). Deep measurement probe was placed directly on gastrocnemius muscle to ensure a deep muscle flow measurement. Ischemic and non-ischemic limb perfusion was measured pre-, post-surgery, 3 days, 7 days and 2 weeks after surgery. The final blood flow values were expressed as the ratio of ischemic to non-ischemic hind limb perfusion.

Post-contraction hyperemia pre- and post- ischemia. After anesthesia, mice were placed on a heated pad. The adductor muscle group and gastrocnemius muscle were exposed by a middle line incision of the limb. After baseline gastrocnemius blood flow was measured, adductor muscles were stimulated with 2 electrodes at 2Hz, 5mA by using electrostimulator for 2min. Blood flow was taken and recorded by MacLab Chart software (ADInstruments, Grand Junction, CO) during stimulation and for 10 minutes post-stimulation.
**Microfil Perfusion.** 4 weeks after femoral ligation, mice were anesthetized and perfused with 20 ml of 37°C PBS plus 10 units/ml heparin at a flow rate of 10–15 ml/min through the left ventricle. After PBS, mice received 20 ml of 4% paraformaldehyde, and 15 ml of Microfil [MV-112 (white), Flowtech, Carver, MA]. The Microfil polymerized overnight at 4°C, and the collagen gels and underlying abdominal musculature were harvested and clarified in graded glycerol solutions (40–100% glycerol in water, increased by 20% glycerol at 24-h intervals). The clarified specimens were viewed on an SMZ1000 dissecting microscope (Nikon).

**Histology and immunohistochemistry.** Mice were sacrificed on day 3, 7 or 14 post-surgery and muscles of the lower limbs were harvested, methanol fixed and paraffin embedded. Tissue sections (5 µm thick) were stained using anti-PECAM-1 (CD31, BD Pharmingen, San Diego, CA), anti smooth muscle alpha actin (SMA) (Dako, Carpinteria, CA), anti-CD45 (BD Pharmingen, rat mAb), anti-PCNA (Santa Cruz, rabbit polyclonal) and anti-nitrotyrosine (Chemicon, rabbit polyclonal) antibodies. Bound primary antibodies were detected using avidin-biotin-peroxidase (NovaRed™ peroxidase substrate kit, Vector Laboratories, Burlingame, CA). TUNEL assay was performed according to the protocol provided by the vendor (Roche). In situ dihydroethidium fluorescence (DHE) was performed as described. Briefly, the unfixed tissues were cut into 10 µM-thick sections, and incubated with 2x10-6mol/dL DHE at 37°C for 30 min in a light-protected humidified chamber. Pictures from 4 random areas of each section, and 5 sections per mice were taken using a Kodak digital camera mounted on a light microscope or equipped with fluorescence microscope (40x objective). Positive staining for each antibody was quantified by measuring the percentage of positive staining/area using the Metamorph software (Molecular Devices, MDS Analytical Technologies).

**Gene expression in ischemic muscle.** Total RNA of lower limb muscles was isolated by using phenol/chloroform and isolated using RNeasy kit with DNase I digestion (Qiagen, Valecia, CA). Reverse transcription was done by standard procedure (Super Script First-Strand Synthesis System, Qiagen) using 1mg total RNA. Quantitative real-time PCR was performed by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (Bio-Rad Laboratories, Inc. Hercules, CA). Specific primers for human and mouse Trx2 were used (human sence primer: 5’-AGG ATC TCC TTG ACA ACC TTT A-3’; mouse sense primer: 5’-AGA
GTC TGT TTG ACG ACC TTT A-3’; both human and mouse reverse primer: 5’-TCA GCC AAT CAG CTT CTT CAG GAA-3’. 18 S ribosomal RNA was used as an internal control. Relative amount of mRNA in lower limb muscles was quantified.

**Immunoblotting and antibodies.** Frozen tissues or MEC after various treatments were lysed by sonication in 1.5 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.75% Brij 96, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM PMSF, 1 mM EDTA) and incubated on ice for 20 min. Protein concentrations were determined with a Bio-Rad kit. The cell lysates were subjected to SDS-PAGE followed by immunoblotting (Immobilon P, Millipore, Milford, MA). The chemiluminescence was detected using an ECL kit according to the instructions of the manufacturer (Amersham Life Science, Arlington Heights, IL). Anti-ASK1, anti-SOD1 and anti-β-tubulin from Santa Cruz; total and phospho-eNOS antibodies from Transduction Laboratory, and anti-Trx1 and Trx2 were generated in our lab.

**Cell culture and cytokines, EC migration assay and image analysis.** Mouse EC isolation from lung tissues was performed as we described previously. Human recombinant TNF and VEGF were from R&D Systems Inc. (Minneapolis, MN) and used at 10 ng/ml. EC migration was modified from the method as described previously. Briefly, EC were cultured in 0.5% FBS overnight and subjected to “wound injury” with a tip. Cells were plated with fresh media and were further cultured for 12-24 h. The EC migration in culture was determined by measuring “wound” distance in cell monolayers. Three different images from each well along the wound were captured by a digital camera under a microscope (4x). A haemocytometer (1mm²/grid) was used as a standard. Wound area (mm) was measured and analyzed by NIH Image 1.60. Statistical analyses were performed with StatView 4.0 package (ABACUS Concepts). Data are presented as means (+SD). Differences were analyzed by unpaired 2-tailed Student t test. Values of p<0.05 were taken as significant.

**Endothelium progenitor cell (EPC) mobilization assay.** After anesthesia and heparinization, blood was drawn by cardiac puncture. 500 µl blood was mixed with 2 ml PBS, gently added to 2 ml Histopaque-1077 (Sigma) and centrifuged at 400g for 30 min. The mononuclear fraction was collected, washed in PBS and
following red cell lysis with Ammonium Chloride solution (StemCell Technologies), \(1 \times 10^6 \text{ cell/cm}^2\) were seeded on fibronectin coated slides (Clontech, San Jose, CA). Cells were allowed to differentiate in EGM-2 SingleQuots medium (Cambrex, East Rutherford, New Jersey) containing VEGF-A, FGF, IGF-1, Hydrocortisone, Ascorbic acid, GA 1000, Heparin, 1% Gentamicin/Streptomycin (GIBCO, Carlsbad, CA) and 5% FBS. Medium was changed every other day. After 5 days of in vitro culture, cells were incubated in 10 \(\mu\text{g/ml}\) Ac-Dil-LDL (Biomedical Technologies) for 4 hours and Ac-Dil-LDL positive cells were counted from digital images obtained at low power (10x).

**Statistical analysis.** All data are expressed as means ± standard error of the mean (SEM). Statistical differences were measured by either Student’s T-test, one or two-way analysis of variance followed by Bonferonni post-hoc test. A value of \(p < 0.05\) was considered as statistically significant.


Supplemental Fig. I

Supplemental Fig. I. Trx2-TG mice show augmented recovery of limb perfusion compared to non-transgenic WT mice. Ischemic hindlimb model was performed, and blood flow of ischemic and non-ischemic limb were measured on gastrocnemius muscle at 30 min, 3, 7 and 14 days after surgery. Blood flow from both non-ischemic (left) and ischemic (right) limbs are shown. N=10 for each strain. Data are mean ± SEM, *, p<0.05 comparing flow on day 3, 7 or 14 to day 0. Open symbols indicates non-ischemic legs and solid symbols for ischemic legs.
Supplemental Fig.II

Critical roles of Trx2 in ischemia-induced arteriogenesis and pericyte recruitment. a. Arteriogenesis was determined by Microfil casting analyses. 2 weeks after femoral ligation (the ligation sites are indicated by arrowheads), mice were anesthetized and subjected to microfil perfusion. Collateral artery sprouting is indicated by arrows. b. Vascular growth was quantified as ratio of vascular density (left/right). n=10 for each strain. *, p<0.05. c-d. Effects of Trx2 on pericyte recruitment. 14 days after femoral ligation, gastrocnemius muscles were harvested. Pericyte recruitment were immunostained with SMA (a smooth muscle/pericyte marker). Representative images of SMA staining are shown in c. SMA-negative and SMA-positive vessels are indicated by arrows and arrowheads, respectively. Quantification of SMA-positive staining (number/mm² muscle area) is shown in d. Data from 3 sections of each mouse muscle tissue are shown in graphics and n=4 for each strain (total 12 sections). *, p<0.05.
Supplemental Fig.III. Regulation of Trx2 expression in ischemic tissues. a-b. WT and Trx2-TG mice (n=4 for each strain) were subjected to hindlimb ischemia, and hindlimbs were harvested on day 0, 3, 7 and 14 post-surgery as indicated. Gene expression of Trx2 was determined by qRT-PCR. 18S rRNA was used for normalization. Normalized Trx2 gene expression (a) and reduction fold (left/right, b) are shown. c. Day 3 samples from 2 mice at each group were used for protein analyses. Trx2, Trx1, SOD1 and SOD2 were determined by Western blot with respective antibodies. β-tubulin was used as a control. Relative levels of Trx2 are shown, with non-ischemic WT as 1.0. n=4. d. Trx2 was induced in vascular endothelium. Trx2 protein in the paraffin sections of non-ischemic and ischemic (day 3) lower limb was detected by immunohistochemistry with anti-Trx2 antibody. Trx2-positive vessels and myocytes are indicated by arrows and arrowheads, respectively. Quantification analyses of Trx2 staining in vessels do not show statistical differences between control and ischemic groups in Trx2-TG mice (10 vessels in each group).
**Supplemental Fig.IV**

**a.** Non-ischemic  Ischemic

WT

TG

**b.** Non-ischemic  Ischemic

WT

TG

**c.**

\[
\text{Leukocytes/mm}^2
\]

\[
\begin{array}{c|cc}
\text{WT} & 0 & 100 \\
\text{TG} & 200 & 600 \\
\end{array}
\]

**d.** Non-ischemic  Ischemic

WT

TG

**e.**

\[
\text{TUNEL-positive cells/PF}
\]

\[
\begin{array}{c|cc}
\text{WT} & 0 & 30 \\
\text{TG} & 10 & 25 \\
\end{array}
\]

**Supplemental Fig.IV.** **Trx2 reduces oxidative responses and inflammation.** WT and Trx2-TG mice were subjected to ischemia ligation, and tissues were harvested on day 7. **a.** Nitrotyrosine, an indicator of peroxynitrite-induced tyrosine nitrosylation, was determined by immunostaining with antibody against Nitrotyrosine. Nitrotyrosine-positive vessels and myocytes are indicated by arrows and arrowheads.
respectively. **b-c.** Recruitment of leukocytes in response to ischemia was determined by anti-CD45. Representative images of non- and ischemic hindlimbs in WT and Trx2-TG are shown. CD45-positive cells were quantitated as number of infiltration/mm² muscle area. **d-e.** Apoptosis in Trx2-TG mice in response to ischemia. Apoptosis in gastronemius was determined by TUNEL assay. TUNEL-positive nuclei of EC, infiltrated cell and myocyte are indicated by black arrows, black arrowheads and white arrows, respectively. Total TUNEL-positive cells were counted as number/mm²(e). Data from different mice groups are shown in graphs. n=4 for each strain. *, p<0.05. **f-g.** Cellular proliferation was determined by PCNA staining. PCNA-positive vessel and muscle fibers are indicated by arrows and arrowheads, respectively. Representative images of ischemic and non-ischemic hindlimbs in WT and Trx2-TG mice are shown. PCNA-positive cells were counted as number/mm². Data from different mice groups are shown in graphs. n=4 for each strain. *, p<0.05.
Supplemental Fig. V. Trx2 expression increases EC migration and EC survival. a. Effects of Trx2 on EC migration. Mouse microvessel EC (MEC) from muscle and lung were isolated from WT and Trx2-TG mice, and the effect of Trx2 and L-NAME on VEGF-induced EC migration was first determined in a monolayer injury model. Representative images are shown. b. Trx2 on EC apoptosis. WT and Trx2-TG MEC were treated with TNF (10 ng/ml)+CHX (10 µg/ml) for 6 h. EC apoptosis was determined by FACS analysis with FITC-conjugated annexin V (X-axis) and propidium iodide (PI, Y-axis). Representative FACS graphs are shown.
Supplemental Fig. VI

Littermates obtained from Trx2-TG/eNOS+/- x Trx2-TG/eNOS+/- mice

<table>
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<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>+/-</td>
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KO (500 bp)  WT (420 bp)  eNOS+/-  eNOS-/-  Trx2-TG/eNOS+/-  Trx2-TG/eNOS-/-

Supplemental Fig. VI. Genotyping of Trx2-TG/eNOS-KO mice. Trx2-TG mice were mated with eNOS+/- mice (both are C57BL/6 background) to obtain Trx2-TG/eNOS+/- mice. Trx2-TG/eNOS+/- mice were further mated with eNOS+/- mice to obtain Trx2-TG/eNOS-KO mice. Mouse tail genomic DNA were genotyped by PCR with specific primers for eNOS-WT, eNOS-KO and Trx2-transgenic fragments. A representative genotyping of littermates from one mother is shown.
**Supplemental Fig. VII**

**a.**

*WT, eNOS-KO, and eNOS-KO/Trx2-TG mice were subjected to ischemia ligation.*

Capillaries and dilated mature vessels are indicated by arrows and arrowheads, respectively (a). Quantification of capillaries (number/mm² muscle area) and ratio of CD31/muscle fiber are shown in b-c. Data from different mice are shown in graphics and n=4 for each strain. *, p<0.05.

**Supplemental Fig. VII. Effects of eNOS deletion on Trx2 augmented ischemia-induced angiogenesis.**