Essential Role of Extracellular SOD in Reparative Neovascularization Induced by Hindlimb Ischemia

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Abstract—Neovascularization is an important physiological repair mechanism in response to ischemic injury, and its process is dependent on reactive oxygen species (ROS). Overproduction of superoxide anion (O$_2^-$) rather contributes to various cardiovascular diseases. The extracellular superoxide dismutase (ecSOD) is one of the major antioxidant enzymes against O$_2^-$ in blood vessels; however, its role in neovascularization induced by tissue ischemia is unknown. Here we show that hindlimb ischemia of mice stimulates a significant increase in ecSOD activity in ischemic tissues where ecSOD protein is highly expressed at arterioles. In mice lacking ecSOD, ischemia-induced increase in blood flow recovery, collateral vessel formation, and capillary density are significantly inhibited. Impaired neovascularization in ecSOD$^{-/-}$ mice is associated with enhanced O$_2^-$ production, TUNEL-positive apoptotic cells and decreased levels of NO$_2^-/NO_3^-$ and cGMP in ischemic tissues as compared with wild-type mice, and it is rescued by infusion of the SOD mimetic tempol. Recruitment of inflammatory cells into ischemic tissues as well as numbers of inflammatory cells and endothelial progenitor cells (c-kit$^+/CD31^+$ cells) in both peripheral blood and bone marrow (BM) are significantly reduced in these knockout mice. Of note, ecSOD expression is markedly increased in BM after ischemia. NO$_2^-/NO_3^-$ and cGMP levels are decreased in ecSOD$^{-/-}$ BM. Transplantation of wild-type BM into ecSOD$^{-/-}$ mice rescues the defective neovascularization. Thus, ecSOD in BM and ischemic tissues induced by hindlimb ischemia may represent an important compensatory mechanism that blunts the overproduction of O$_2^-$, which may contribute to reparative neovascularization in response to ischemic injury. (Circ Res. 2007;101:409-419.)

Key Words: superoxide dismutase ■ reactive oxygen species ■ neovascularization ■ bone marrow ■ endothelial progenitor cells

Neovascularization is important repair mechanism to preserve tissue integrity in response to ischemic injury. It is a key process involved in normal development and wound repair as well as in the various pathophysiologicals such as ischemic heart and limb diseases. New blood vessels grow postnatally via angiogenesis and arteriogenesis.1,2 Moreover, circulating endothelial progenitor cells (EPCs) mobilized from bone marrow (BM) also contribute to the formation of new blood vessels (vasculogenesis) after tissue ischemia.3,4 Inflammation is an early key event required for ischemia-induced neovascularization.5,6 Reactive oxygen species (ROS) such as superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are involved in physiological and pathophysiological responses. Excess amounts of ROS contribute to the pathogenesis of many cardiovascular diseases by inducing apoptosis and cell death.7 In contrast, physiological levels of ROS produced by growth factor and tissue ischemia are involved in proliferation and migration of endothelial cells (ECs), thereby contributing angiogenesis in vivo.7 Indeed, we previously demonstrated that hindlimb ischemia stimulates ROS production via gp91phox (Nox2)-based NADPH oxidase in ischemic tissues, and that blood flow recovery are markedly impaired in Nox2$^{-/-}$ mice in which ischemia-induced ROS production is abolished.8 In contrast, overproduction of Nox2-derived ROS rather contributes to impairment of posts ischemic neovascularization in pathological conditions such as diabetes.9 Thus, function of ROS during new blood vessel formation is dependent on its concentration.

Levels of ROS are tightly controlled by the balance of activity of pro- and antioxidative enzymes. The extracellular superoxide dismutase (ecSOD) is the major antioxidant enzyme against O$_2^-$ in the extracellular space where ROS are accumulated.10,11 It is synthesized and secreted by vascular smooth muscle cells and fibroblasts, and anchored to the extracellular matrix and endothelial cell surface through binding to the heparan sulfate proteoglycan, collagen, and fibulin-5.12,13 Because of its location, ecSOD plays an important role in regulating endothelial function by modulating the
levels of $O_2^-$ and bioavailability of nitric oxide (NO)\textsuperscript{14–16}. We and others reported that ecSOD expression is influenced by multiple stimuli, including nitric oxide and proinflammatory cytokines.\textsuperscript{17,19} Previous studies using ecSOD$^{-/-}$ mice demonstrate that ecSOD functions to prevent oxidative stress–dependent pathological states such as ischemia–reperfusion injury and lung injury induced by hyperoxia.\textsuperscript{12,17} In some of these models, protective effects of ecSOD are associated with a decrease in recruitment of inflammatory cells to the injury sites.\textsuperscript{19,20} Moreover, adenovirus-mediated delivery of ecSOD inhibits growth of B16 melanoma tumors by blocking neovascularization in mice.\textsuperscript{21}

We performed the present study to determine whether endogenous ecSOD is involved in neovascularization in response to hindlimb ischemia by modulating the levels of $O_2^-$ using ecSOD$^{-/-}$ mice. We demonstrate that hindlimb ischemia of mice increases expression of ecSOD in ischemic tissues and BM. Mice lacking ecSOD show enhanced ischemia-induced $O_2^-$ production, decreased NO$_3^-/NO_2^-$ and cyclic GMP levels, increased apoptosis in ischemic tissues, which may contribute to impairment of neovascularization. Numbers of inflammatory cells and EPCs in both peripheral blood and BM are reduced in ecSOD$^{-/-}$ mice, which is associated with decrease in BM cells differentiation into EPCs as well as levels of NO$_3^-/NO_2^-$ and cyclic GMP in BM. Defective neovascularization in ecSOD$^{-/-}$ mice is rescued by SOD mimetic infusion as well as by transplantation of BM from WT mice. These findings suggest that ecSOD plays an essential role in reparative neovascularization in response to ischemic injury by protecting ischemic tissues and BM from overproduction of ROS.

Materials and Methods

**Mouse Ischemia Hindlimb Model**

The ecSOD-deficient mice in a C57Blk/6 background and C57Blk/6 (wild-type) mice were used for this study.\textsuperscript{15} Surgical procedure are performed as previously described (see the online data supplement, available at http://circres.ahajournals.org).\textsuperscript{8}

**Laser Doppler Blood Flow Analysis**

We measured hindlimb blood flow using a laser Doppler blood flow (LDBF) analyzer (PIM III, Perimed) as previously described (see the online data supplement).\textsuperscript{8}

**Blood Flow Measurement by Microsphere**

Fluorescent microspheres (15-$\mu$m diameter, 5$x10^6$ heads, Molecular Probe) were used to analyze the formation of conductance collateral vessels as previously described (see the online data supplement).\textsuperscript{8}

**Immunocytochemical Analysis**

Staining for capillary, proliferating arterioles, inflammatory leukocytes, and macrophages are performed as previously described (see also the online data supplement).\textsuperscript{8}

**Microcomputed Tomography (micro-CT) Analysis**

Tissues were prepared, and analyzed as previously described (see the online data supplement).\textsuperscript{22}

**Measurement of ecSOD Activity**

The ecSOD activity was measured by cytochrome $c$ reduction assay after separation of ecSOD fraction with ConA-Sepharose column as described in the online data supplement.\textsuperscript{23}

**Measurements of Superoxide ($O_2^-$) Production in Hindlimb Tissues**

$O_2^-$ production was measured with 5 $\mu$mol/L lucigenin-enhanced chemiluminescence, as previously described.\textsuperscript{8} As a second approach to measure $O_2^-$ in hindlimb tissues in situ, dihydroethidium (DHE) were used as previously described.\textsuperscript{13}

**Measurement of NO$_3^-/NO_2^-$ and cGMP Production**

Levels of the nitrite [NO$_2^-$] plus nitrate [NO$_3^-$] were measured by the Griess method (Cayman), according to manufacturer’s protocol. Tissue cGMP was measured using a cGMP enzyme-immunoassay system (Amersham Life Science).

**FACS Analysis**

Fluorescence-activated cell sorter (FACS) analysis was used to quantify inflammatory cells and EPC-like mononuclear cells (MNCs) in both peripheral blood (PB) and bone marrow (BM) from WT and ecSOD$^{-/-}$ mice as described in the online data supplement.\textsuperscript{24}

**BM Transplantation**

BM transplantation was performed as described in the online data supplement.

**Statistical Analyses**

All values were expressed as mean±SE. Blood flow recovery in the ischemic hindlimb was compared between 2 groups by 2-way repeated measures ANOVA, followed by Bonferroni post hoc analysis. Comparison between 2 mean values was evaluated by an unpaired Student 2-tailed $t$ test, and between 3 or more groups was evaluated by 1-way ANOVA followed by Bonferroni post hoc analysis. Statistical significance was accepted at $P<0.05$.

**Results**

**Ischemia-Induced Neovascularization Is Impaired in ecSOD$^{-/-}$ Mice**

All mice survived after induction of unilateral hindlimb ischemia, appeared to be healthy, and showed no significant change of blood pressure and heart rate during the follow-up period. To determine the role of ecSOD in ischemia-induced neovascularization, we measured blood flow recovery in ischemic and nonischemic limbs after ligation of femoral artery in WT and ecSOD$^{-/-}$ mice. Figure 1A using LDBF analysis shows that in WT mice, hindlimb blood flow was markedly decreased immediately after surgery, partially restored on day 3, and recovered to the level of that of the nonischemic limb by day 7. In ecSOD$^{-/-}$ mice, ischemia-induced blood flow recovery was delayed, and the LDBF ratio at 7 days after ischemia was significantly decreased as compared with that in WT mice. Similar precipitous reduction in hindlimb flow occurred in WT and ecSOD$^{-/-}$ mice, indicating that the severity of ischemia was similar in both groups.

Neovascularization induced by tissue ischemia is mediated through arteriogenesis and angiogenesis.\textsuperscript{1} We thus examined the role of ecSOD in ischemia-induced arteriogenesis using microspheres of 15 $\mu$m diameter to analyze the formation of conductance collateral vessels.\textsuperscript{8} Figure 1B shows that the
recovery of collateral blood flow at 7 days after ischemia was significantly reduced in ecSOD\textsuperscript{+/−} mice compared with WT mice. Consistent with this result, immunofluorescence analysis in collateral vessels revealed that the number of BrdU positive arterioles was decreased in ischemic hindlimbs in ecSOD\textsuperscript{+/−} mice (Figure 1C). To confirm further, we performed micro-CT analysis and found that collateral formation was markedly reduced in ecSOD\textsuperscript{+/−} mice compared with WT mice. Consistent with this result, immunofluorescence analysis in collateral vessels revealed that the number of BrdU positive arterioles was decreased in ischemic hindlimbs in ecSOD\textsuperscript{+/−} mice (Figure 1C). To confirm further, we performed micro-CT analysis and found that collateral formation was markedly reduced in ecSOD\textsuperscript{+/−} mice compared with WT mice.
mice (supplemental Figure I). Taken together, arteriogenesis is impaired in ecSOD−/− mice.

To determine the role of ecSOD in ischemia-induced angiogenesis, we measured capillary density by staining ischemic tissue with *Griffonia simplicifolia* lectin which detects ECs with high efficiency in our system.8 Figure 1D shows that lectin-positive capillary density was significantly reduced in the ischemic adductor muscle of ecSOD−/− mice compared with WT mice.

**ecSOD Activity and Protein Expression in Ischemic Tissues Are Increased in Response to Hindlimb Ischemia**

We next examined the ecSOD activity and protein expression after hindlimb ischemia. Figure 2A shows that ecSOD activity in ischemic tissues as measured by cytochrome c reduction assay was significantly increased after femoral artery ligation. Immunocytochemistry with double staining for ecSOD and α-actin or lectin shows that ecSOD protein was highly expressed at α-actin positive arterioles, not at lectin-positive capillary-like ECs, in ischemic tissues (Figure 2B). We also found that ecSOD is expressed in inflammatory cells such as macrophage (Mac3 positive) (supplemental Figure II).

**Ischemia-Induced O$_2^{-}$ Production Is Enhanced in ecSOD−/− Mice**

Because ecSOD is one of the major antioxidant enzymes against O$_2^{-}$ in the vasculature, we examined O$_2^{-}$ levels in ischemic and nonischemic limbs in WT- and ecSOD−/− mice.
Figure 3A using lucigenin assays demonstrate that hindlimb ischemia stimulates $O_2^\cdot$ production in ischemic tissues at 7 days after operation in WT mice. Mice deficient in ecSOD show enhanced ischemia-induced, but not basal, $O_2^\cdot$ production as compared with WT mice, suggesting a protective role of ecSOD from overproduction of ROS induced by ischemic injury. To confirm this result further, we performed DHE staining which is specific for $O_2^\cdot$ in ischemic and nonischemic hindlimb tissue sections. Figure 3B shows an increase of DHE fluorescence in ischemic tissue in WT mice, which was further enhanced in ecSOD KO mice. Of note, an increase in DHE staining was almost completely abolished by coinubation with SOD, demonstrating ischemia-induced increase in $O_2^\cdot$ in WT mice and its augmentation in ecSOD KO mice.

**Chronic Tempol Infusion Rescues Impairment of Ischemia-Induced Neovascularization in ecSOD KO Mice**

To determine whether overproduction of ROS contributes to impairment of postischemic neovascularization in ecSOD KO mice, we infused the SOD mimetic tempol into WT and ecSOD KO mice, and measured $O_2^\cdot$ production and blood flow recovery. Tempol treatment into WT mice significantly reduced the ischemia-induced flow recovery and $O_2^\cdot$ level in ischemic tissues (Figure 4A) at 7 days after operation (Figure 4B). In ecSOD KO mice, treatment with tempol rather rescued the impairment of ischemia-induced blood flow recovery (Figure 4B) by reducing the overproduction of $O_2^\cdot$ to the levels observed in WT mice without tempol after hindlimb ischemia.
ischemia (Figure 4A). These results are consistent with the notion that optimal levels of ROS are required but excess amount of ROS are inhibitory for neovascularization induced by tissue ischemia.

Decrease in NO$_2^-$/NO$_3^-$ and cGMP Levels and Increase in Apoptosis in ecSOD$^{-/-}$ Mice

It has been shown that ecSOD preserves bioavailable NO by preventing the reaction of NO with excess amount of O$_2^-$. NO also plays an important role in postnatal neovascularization. To assess the mechanisms by which overproduction of O$_2^-$ by ecSOD deficiency inhibits neovascularization, we measured NO$_2^-$/NO$_3^-$ and cGMP levels in ischemic tissues from WT and ecSOD$^{-/-}$ mice. Figure 4C shows that NO$_2^-$/NO$_3^-$ and cGMP levels in ischemic tissue were markedly decreased in ecSOD$^{-/-}$ mice as compared with WT mice. Of note, their reduction in ecSOD$^{-/-}$ mice was restored by tempol treatment (data not shown). Moreover, we found that the numbers of apoptotic cells as measured by TUNEL staining were markedly increased in ischemic tissues in ecSOD$^{-/-}$ mice as compared with WT mice (supplemental Figure III). These results suggest that overproduction of O$_2^-$ results in decrease in NO/cGMP levels and increased apoptosis, which may contribute to defective neovascularization in ecSOD$^{-/-}$ mice.

Numbers of Inflammatory Cells in Ischemic Sites as Well as in Peripheral Blood and Bone Marrow Are Decreased in ecSOD$^{-/-}$ Mice

Because inflammation plays a key role in ischemia-induced neovascularization, we examined the number of inflammatory cells infiltrated into the ischemic hindlimbs in WT and ecSOD$^{-/-}$ mice. Figure 5 using immunocytochemical analysis shows that the numbers of infiltrated CD45 positive leukocytes and Mac3-positive macrophages were significantly decreased in ischemic tissue from ecSOD$^{-/-}$ mice at 3 days after ischemia. FACS analysis of peripheral blood (PB) and bone marrow (BM) reveals that there was significant decrease in the numbers of CD45 positive neutrophils and monocytic cells in PB (Figure 6A) as well as CD45 positive myeloid cells in BM (Figure 6B) in ecSOD$^{-/-}$ mice compared with WT mice.

EPC-Like Cells in Peripheral Blood and Bone Marrow Are Decreased in ecSOD$^{-/-}$ Mice

Because postnatal neovascularization is also dependent on vasculogenesis, we examined the number of c-kit$^+$/CD31$^+$ EPC-like cells in PB and BM in WT and ecSOD$^{-/-}$ mice. FACS analysis shows a significant reduction in the number of c-kit$^+$/CD31$^+$ cells in both PB (Figure 7A) and BM (Figure 7B) at 3 days after ischemia in ecSOD$^{-/-}$ mice compared with WT mice.

NO$_2^-$/NO$_3^-$ and cGMP Levels in BM as Well as BM-MNC Differentiation Into EPCs are Decreased in ecSOD$^{-/-}$ Mice

Above results suggest that BM function is impaired in ecSOD$^{-/-}$ mice. To assess underlying mechanisms, we examined NO$_2^-$/NO$_3^-$ and cGMP levels in BM cells as well as capacity of BM-MNC differentiation into EPCs in WT- and ecSOD$^{-/-}$ mice. Figure 7C shows that both NO$_2^-$/NO$_3^-$ and cGMP levels were significantly lower in BM from ecSOD$^{-/-}$ mice than that from WT mice. As shown in Figure 7D, BM culture assay reveals that differentiation of BM-MNCs into EPCs, as detected by Dil-acLDL and BS lectin double positive cells, was markedly decreased in ecSOD$^{-/-}$ mice.
Neovascularization in ecSOD

For BM function, thereby regulating postnatal ischemia. Taken together, these results suggest that ecSOD shows the significant decrease in blood flow recovery after ischemia. We found that: (1) Hindlimb ischemia of mice rescued the impairment of blood flow recovery after hindlimb ischemia and performed BM transplantation between WT and ecSOD mice. Moreover, WT mice transplanted with BM from ecSOD mice rescued the defective blood flow recovery after hindlimb ischemia in ecSOD mice. WT mice rescued the impairment of blood flow recovery after hindlimb ischemia and performed BM transplantation between WT and ecSOD mice. Moreover, WT mice transplanted with BM from ecSOD mice rescued the defective blood flow recovery after hindlimb ischemia in ecSOD mice. WT mice rescued the impairment of blood flow recovery after hindlimb ischemia and performed BM transplantation between WT and ecSOD mice. Moreover, WT mice transplanted with BM from ecSOD mice rescued the defective blood flow recovery after hindlimb ischemia in ecSOD mice. WT mice rescued the impairment of blood flow recovery after hindlimb ischemia and performed BM transplantation between WT and ecSOD mice. Moreover, WT mice transplanted with BM from ecSOD mice rescued the defective blood flow recovery after hindlimb ischemia in ecSOD mice. WT mice rescued the impairment of blood flow recovery after hindlimb ischemia and performed BM transplantation between WT and ecSOD mice. Moreover, WT mice transplanted with BM from ecSOD mice rescued the defective blood flow recovery after hindlimb ischemia in ecSOD mice. WT mice rescued the impairment of blood flow recovery after hindlimb ischemia and performed BM transplantation between WT and ecSOD mice. Moreover, WT mice transplanted with BM from ecSOD mice rescued the defective blood flow recovery after hindlimb ischemia in ecSOD mice.

**Discussion**

The present study provides novel evidence that endogenous ecSOD plays an essential role for postischemic neovascularization. We found that: (1) Hindlimb ischemia of mice stimulates a significant increase in ecSOD activity in ischemic tissues where ecSOD protein is highly expressed at arterioles and in part at inflammatory cells; (2) Mice lacking ecSOD show overproduction of \( \text{O}_2^- \) in ischemic tissues, which is associated with decreased \( \text{NO}_2^-/\text{NO}_3^- \) and cGMP levels as well as increased apoptosis, thereby contributing to impairment of neovascularization; (3) Recruitment of inflammatory cells into ischemic tissues as well as number of inflammatory cells and EPCs in both PB and BM are significantly decreased in ecSOD−/− mice; (4) ecSOD−/− BM is dysfunctional because \( \text{NO}_2^-/\text{NO}_3^- \) and cGMP levels in BM as well as BM-MNC differentiation into EPCs are decreased in ecSOD−/− mice; (5) Ischemia increases ecSOD expression in BM, and BM transplantation of WT-BM into ecSOD−/− mice rescues the defective blood flow recovery.

We demonstrate that ecSOD activity is significantly increased in the ischemic tissues after hindlimb ischemia, which is associated with an increase in ROS production and neovascularization. Immunocytochemial analysis reveals that ecSOD protein is predominantly expressed at \( \alpha \)-actin positive arterioles and in part at mac3-positive inflammatory cells, but not at lectin-positive capillary-like ECs. This is consistent with the previous reports that ecSOD is highly expressed in vascular smooth muscle and inflammatory cells, but not in endothelial cells. Functional role of upregulation of ecSOD in neovascularization is demonstrated by the observation that ischemia-induced increase in blood flow recovery and capillary density (angiogenesis) are significantly inhibited in ecSOD−/− mice. Moreover, formation of collateral artery (arteriogenesis) as measured by microsphere (15 \( \mu \)m diameter), number of BrdU-positive arterioles, and micro-CT are significantly impaired in ecSOD−/− mice. These results suggest that endogenous ecSOD plays an important role in ischemia-induced neovascularization.

**ecSOD+/− Bone Marrow Rescues Impairment of Neovascularization in ecSOD−/− Mice**

To assess the role of ecSOD in BM function in vivo, we examined the ecSOD protein expression in BM in response to hindlimb ischemia and performed BM transplantation between WT and ecSOD−/− mice. Western analysis shows that ecSOD protein expression was markedly increased in the BM at 3 and 7 days after ischemia while Cu/Zn SOD protein was not changed (Figure 8A). Figure 8B shows that WT, but not ecSOD−/−, BM transplantation to the irradiated ecSOD−/− mice rescued the impairment of neovascularization. Immunocytochemial analysis reveals that ecSOD protein is predominantly expressed at arterioles and in part at inflammatory cells; (2) Mice lacking ecSOD show overproduction of \( \text{O}_2^- \) in ischemic tissues, which is associated with decreased \( \text{NO}_2^-/\text{NO}_3^- \) and cGMP levels as well as increased apoptosis, thereby contributing to impairment of neovascularization; (3) Recruitment of inflammatory cells into ischemic tissues as well as number of inflammatory cells and EPCs in both PB and BM are significantly decreased in ecSOD−/− mice; (4) ecSOD−/− BM is dysfunctional because \( \text{NO}_2^-/\text{NO}_3^- \) and cGMP levels in BM as well as BM-MNC differentiation into EPCs are decreased in ecSOD−/− mice; (5) Ischemia increases ecSOD expression in BM, and BM transplantation of WT-BM into ecSOD−/− mice rescues the defective blood flow recovery.

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In contrast, Wheeler et al reported that overexpression of ecSOD using adenovirus inhibits tumor angiogenesis. This discrepancy may be attributable to the difference of angiogenesis model system; i.e., hindlimb ischemia and B16 melanoma.

**Figure 6.** The numbers of inflammatory cells in peripheral blood PB (A) and bone marrow BM (B) after hindlimb ischemia are decreased in ecSOD−/− mice. Upper panel, the number of CD45-positive cells at 3 days after hindlimb ischemia in PB (A) and BM (B) from WT and ecSOD−/− mice, assessed by FACS analysis. Lower panel, statistical analysis of CD45 positive cells within the marked region in PB (neutrophil and monocytic cell population) and BM (myeloid cell population) (n=6, *P<0.05 vs WT).
Lucigenin assay and DHE fluorescence analysis demonstrate that ischemia-induced $O_2^\cdot$ production in ischemic tissue is further enhanced in ecSOD $^{-/-}$ mice compared with WT mice. Infusion of the SOD mimetic tempol into ecSOD $^{-/-}$ mice rescues the impairment of ischemia-induced blood flow recovery by reducing the overproduction of $O_2^\cdot$ to the levels observed in WT mice. These results suggest that overproduction of ROS attributable to ecSOD deficiency contributes to the impairment of ischemia-induced neovascularization. Consistent with this, Ebrahimian et al reported that excess amount of ROS impairs postischemic neovascularization in pathological conditions such as type1 diabetes. By contrast, WT mice treated with tempol inhibits neovascularization with concomitant decrease in $O_2^\cdot$ production, suggesting that optimal levels of ROS are required for neovascularization. Indeed, we and others previously demonstrated that ROS are essential for angiogenesis signaling in cultured endothelial cells and postnatal neovascularization in vivo. Thus, ischemia-induced upregulation of ecSOD expression may represent an important compensatory mechanism that protects ischemic tissues from overproduction of $O_2^\cdot$, thereby preserving appropriate level of ROS required for new blood vessel formation. Moreover, our findings are consistent with the notion that optimal low levels of ROS are required but excess amounts of ROS are inhibitory for neovascularization induced by tissue ischemia.

We and others previously demonstrated that ecSOD deficiency is associated with a decrease in NO bioavailability and endothelial dysfunction caused by a rapid reaction of $O_2^\cdot$ and NO. NO also plays an important role in postnatal neovascularization. The present study demonstrates that $NO_2^-/NO_3^-$ and cGMP levels in ischemic tissue are markedly

Figure 7. The numbers of EPC-like cells in PB (A) and BM (B) after hindlimb ischemia, $NO_2^-/NO_3^-$ and cGMP levels in BM (C), and BM-MNC differentiation into EPCs (D) are decreased in ecSOD $^{-/-}$ mice. PB (A) and BM (B) from WT and ecSOD $^{-/-}$ mice were collected at 3 days after ischemia, and EPC-like cells were identified as c-kit and CD31– double-positive cells by FACS analysis (n=6, *$P<0.05$ vs WT). C, Levels of $NO_2^-/NO_3^-$ and cGMP in BM from WT and ecSOD $^{-/-}$ mice. *$P<0.05$ vs WT, D, BM culture assay. The number of Dil-acLDL and BS lectin double positive EPCs measured at 4 days after BM-MNCs from WT and ecSOD $^{-/-}$ mice being cultured in fibronectin-coated plate. *$P<0.05$ vs WT.
decreased in ecSOD−/− mice, which are restored by tempol treatment. Moreover, TUNEL-positive apoptotic cells are markedly increased in ischemic tissues in ecSOD−/− mice as compared with WT mice. Consistent with this, high concentrations of ROS have been shown to cause apoptosis and cell death. Taken together, these results suggest that overproduction of O2− induced by ecSOD knockout results in decreased available NO and increased apoptosis, thereby contributing to impairment of neovascularization. Moreover, it is possible that ecSOD deficiency may cause formation of peroxynitrite, which causes endothelial NO synthase uncoupling to produce excess amount of O2−. The detailed relationships between ecSOD and NO or associated reactive nitrogen species in postnatal neovascularization require further investigation.

Inflammation is a key early process responsible for ischemia-induced neovascularization. The present study demonstrates that ecSOD deficiency inhibits infiltration of inflammatory leukocytes (CD45 positive) and macrophage (Mac3 positive) into ischemic limbs at 3 days after tissue ischemia. Given that infiltrated inflammatory leukocytes release cytokines and angiogenic factors including VEGF, it is conceivable that reduced inflammatory responses in ecSOD−/− mice may contribute to impairment of neovascularization. This is in contrast to the previous reports suggesting an anti-inflammatory function of ecSOD in other models including lung injury induced by various stimulants such as LPS and hyperoxia. This discrepancy may be attributable to the difference of mechanism of inflammation between ischemia-induced angiogenesis and lung injury. Importantly, the present study demonstrates that the number of inflammatory cells in both PB and BM after hindlimb ischemia is significantly reduced in ecSOD−/− mice. Thus, the decrease of inflammatory cells in ischemic tissue induced by ecSOD deficiency may be at least in part attributable to the impairment of BM function which is important for maintenance and mobilization of inflammatory cells.

BM is one of the major reservoirs of EPCs. In response to ischemic injury, EPCs are mobilized from BM and homes to sites of injury, thereby contributing to neovascularization. Flow cytometry analysis shows that the numbers of EPC-like c-kit+/CD31+ cells in both PB and BM are significantly decreased in ecSOD−/− mice. To assess the mechanism by which the numbers of EPCs and inflammatory cells are decreased in ecSOD−/− BM, we examined NO2−/NO3− and cGMP levels in BM cells in WT- and ecSOD−/− mice. Both NO2−/NO3− and cGMP levels are significantly lower in ecSOD−/− BM compared with those in WT-BM. Aicher et al reported that eNOS deficiency in the BM microenvironment impairs the mobilization of stem and progenitor cells from the BM. It has been shown that the local BM microenvironment, so-called stem cell niche, which includes macrophages, fibroblasts, endothelial cells, and extracellular matrixes is important for hematopoiesis and EPC differentiation. In the present study, BM culture assay reveals that differentiation of BM-MNCs into EPCs, as detected by Dil-acLDL and BS lectin double positive cells, is markedly decreased in ecSOD−/− mice. In line with our finding, previous studies show that EPCs are enriched in antioxidant enzymes which are protective against oxidative stress to maintain their function required for postischemic neovascularization, and that overproduction of ROS in the setting of diabetes impairs BM-MNCs differentiation into EPCs. Thus, ecSOD may play an important role in BM function by preserving NO availability in the BM microenvironment from excess amounts of ROS.

To assess the role of ecSOD in BM function, we examined the ecSOD protein expression in BM in response to hindlimb ischemia and performed BM transplantation between WT and ecSOD−/− mice. We demonstrate that ecSOD protein expres-

Figure 8. Transplantation of BM from wild-type mice rescues the impairment of neovascularization in ecSOD−/− mice. A, Western analysis for ecSOD and Cu,Zn-SOD protein expression in BM cells from WT and ecSOD−/− mice at day 0, 3, 7 after hindlimb ischemia (n=3, **P<0.01 vs Day 0). B, The irradiated recipient WT or ecSOD−/− mice were transplanted with BM cells from WT or ecSOD−/− mice and subject to femoral artery ligation at 4 weeks after BM transplantation (n=6, *P<0.05, **P<0.01). Mice were subjected for the measurement of LDBF analysis as described.
sion is markedly increased in the BM after ischemia whereas Cu/ZnSOD protein is not changed. Moreover, impaired blood flow recovery in ecSOD−/− mice is rescued by reception of WT-BM. WT mice transplanted with ecSOD−/− BM show significant decrease in ischemia-induced restoration of blood flow. These data strongly suggest that ischemia-induced upregulation of ecSOD in BM is involved in preserving BM function, thereby promoting neovascularization. Understanding the role of ecSOD in BM cells in neovascularization is the subject of future investigation.

In summary, the present study demonstrates that ecSOD plays a critical role in neovascularization in response to ischemic injury. Ischemia-induced upregulation of ecSOD in BM and ischemic hindlimbs may represent a novel compensatory mechanism that protects against overproduction of ROS, thereby maintaining ROS levels which are required for reparative neovascularization induced by tissue ischemia. These findings provide novel insight into ecSOD as a potential therapeutic target for ischemic cardiovascular diseases. Moreover, our present study using ecSOD−/− mice and previous studies using Nox2−/− mice8,9 support the concept for a double-edged role of ROS in postischemic neovascularization; optimal low levels of ROS are required but excess amounts of ROS are inhibitory for new blood vessel formation.

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Disclosures

None.

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Materials and methods

Mouse Hindlimb Ischemia Model. The ecSOD-deficient mice backcrossed to the C57Blk/6 background for 10 generations were weaned at 4 weeks of age and maintained on regular chow for 3 months. C57Blk/6 (wild-type) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and were fed regular chow. Diet and water were provided ad libitum. Mice were subjected to unilateral hindlimb surgery under anesthesia with intraperitoneal administration of ketamine (87 mg/kg) and xylazine (13 mg/kg). The right femoral artery was exposed, ligated proximally and distally with 5-0 silk ligatures, and excised. In some experiments, the SOD mimetic 4-hydroxy-TEMPO (tempol, Sigma Aldrich) in H2O, 20 mg kg⁻¹ d⁻¹ or saline was chronically infused by osmotic minipump (Alzet model 2002; Alza Corp) from 1 day before operation. The Animal Care and Use Committee of the Emory University School of Medicine and the University of Illinois at Chicago approved study protocols.

Laser Doppler Blood Flow Analysis. We measured hindlimb blood flow using a laser Doppler blood flow (LDBF) analyzer (PIM III, Perimed) as described previously. Mice were anesthetized and placed on a heating plate at 37°C for 10 minutes to minimize temperature variation. Before surgery and on postoperative days 0, 3, and 7, LDBF analysis was performed on legs and feet. Blood flow was displayed as changes in the laser frequency, represented by different color pixels. The mean LDBF values were calculated, and hindlimb blood flow was expressed as the ratio of ischemic to nonischemic LDBF.

Blood Flow Measurement by Microsphere. Mice were euthanized, and fluorescent microspheres (15-µm diameter, 5x10⁵ beads, Molecular Probe) were injected into the left ventricle and then flushed with heparinized saline as described previously with minor modification. Tissues were dissolved in 4 mol/L KOH and filtered with membrane filters (3-µm pore, MILLIPORE). The fluorescent dye was extracted with xylene, and fluorescence was measured with a CytoFluor 3000 plate reader (Applied Biosystems) and normalized by muscle weight.
Microcomputed Tomography (micro-CT) analysis. Tissues were prepared as previously described. After the animals were euthanized, the vasculature was flushed with 0.9% normal saline containing heparin sodium (100 U/ml) at a pressure of ~100 mmHg via a needle inserted into the left ventricle. The specimens were then pressure fixed with 10% neutral buffered formalin. Formalin was flushed from the vessels using heparinized saline, and the vasculature was injected with either a 15% barium sulfate, 2% gelatin suspension or a radiopaque silicone rubber compound containing lead chromate (Microfil MV-122, Flow Tech; Carver, MA). Mouse hindlimbs were dissected from the specimens and soaked for 4 days in 10% neutral buffered formalin to ensure complete tissue fixation. Tissues were subsequently treated for 48 h in a formic acid based solution, Cal Ex II (Fisher Scientific; Pittsburgh, PA), to decalcify the bone and facilitate image thresholding of the hindlimb vasculature from the surrounding tissues. Samples were rinsed thoroughly, soaked for 1 h in water, and then stored at 4°C in 10% neutral buffered formalin. Micro-CT imaging and parametric analysis were performed as previously described.

Immunocytochemical Analysis: Staining for capillary, proliferating arterioles, apoptotic cells, inflammatory leukocytes and macrophages. Tissue sections (7-µm-thick) prepared from OCT-embedded tissue of the ischemic and control hindlimbs were used for immunocytochemical analysis. To quantify proliferating arterioles, mice were injected intraperitoneally with the thymidine analog 5-bromo-2’-deoxyuridine (BrdU; 40 mg/kg body weight, 500 µl, Sigma-Aldrich) at 12 hours and 1 hour before sacrifice. Mice were euthanized and perfused through the left ventricle with saline, and hindlimb muscles were embedded in OCT compound (Sakura Finetek) and snap-frozen in liquid nitrogen. Sections were incubated with a rat anti-BrdU antibody (Abcam) to detect proliferating cells, followed by Rhodamine Red X-conjugated (RRX) goat anti-rat IgG (Jackson ImmunoResearch Laboratories), and a monoclonal mouse anti-smooth muscle α-actin (clone 1A4, Sigma-Aldrich) to detect smooth muscle cells, followed by FITC-conjugated goat antibodies to murine IgG (Jackson ImmunoResearch Laboratories). For nuclear staining, BrdU-labeled tissue sections were counterstained with 4’, 6-diamidino-2-phenylindole
(DAPI). For quantification, the number of BrdU positive arterioles were counted in 5 randomly selected high-power fields (magnification x400) with 3 independent samples. To quantify capillary density within the thigh adductor muscle, tissue slices (7-µm-thick frozen sections) were stained with biotinylated *Griffonia simplicifolia* lectin (Vector Laboratories) for 1 hour at room temperature, followed by fluorescein isothiocyanate (FITC)-conjugated streptavidin (Jackson ImmunoResearch Laboratories) as previously described.² Apoptosis in skeletal muscle was detected by transferase-mediated dUTP nick-end labeling (TUNEL) staining (Roche), according to the manufacturer’s protocol. Inflammatory cells were stained with anti-mouse CD45 antibody (PharMingen) and macrophage was stained with anti-mouse Mac3 antibody (Accurate Chemical & Scientific Corp). The slides were then stained with species specific Rhodamine Red-X- or FITC-conjugated secondary antibody raised against whole IgG (Jackson ImmunoResearch Laboratories). For quantification, the numbers of lectin-, TUNEL-, CD45- or mac3-positive cells were counted in 5 randomly selected high-power fields (magnification x400) with 3 independent samples.

**Measurement of ecSOD activity.** Hindlimb tissues were homogenized in 10 vol of 50 mM potassium phosphate (pH 7.4) containing 0.3 M KBr and a cocktail of protease inhibitors (0.5 mM PMSF, 3 mM diethylene-triaminepentaacetic acid, 90 mg/liter of aprotinin, 10 mg/liter of pepstatin, 10 mg/L of chymostatin, and 10 mg/L of leupeptin). The homogenates were then sonicated and extracted at 4C for 30 min. The extracts were then centrifuged at 3,000 g for 15 min. SOD activity was assayed by monitoring the inhibition of the rate of xanthine oxidase-mediated reduction of cytochrome c (pH 7.4), as described previously.⁶

**Measurements of Superoxide (O₂⁻) Production in Hindlimb Tissues.** O₂⁻ production by ischemic and nonischemic hindlimb tissues was measured by lucigenin-enhanced chemiluminescence assay, as previously described.² Hindlimb muscle samples were placed in scintillation vials containing Krebs-HEPES buffer with 5 µmol/L lucigenin. Light emission was detected with a scintillation counter programmed in out-of-coincidence mode. Mean
chemiluminescence yields observed during a period of 20 minutes after addition of the tissues were used to estimate rates of production of $O_2^{-}$.

As a second approach to measure $O_2^{-}$ in hindlimb tissues in situ, frozen sections (30 µm) of ischemic and nonischemic tissues were stained with fluorophores sensitive to $\cdot$O$_2^-$, dihydroethyldium (DHE) (Molecular Probes, Eugene, OR) as previously described. The specificity of DHE signals for $\cdot$O$_2^-$ detection was confirmed by preincubation with SOD (500 U/mL, Sigma).

**Measurement of Nitrite/nitrate and cGMP production.** Levels of the nitrite [NO$_2^-$] plus nitrate [NO$_3^-$] were measured by the Griess method (Cayman), according to manufacturer’s protocol. Thigh skeletal muscles were harvested from hindlimbs of WT and ecSOD$^{-/-}$ mice (n=5 from each group) at 7 days after ischemia. Samples were weighed, snap-frozen in liquid N$_2$, and stored at −80°C. Tissue cGMP was measured using a cGMP enzyme-immunoassay system (Amersham Life Science).

**FACS Analysis.** To quantify inflammatory cells and EPC-like cells in both peripheral blood (PB) and bone marrow (BM), WT and ecSOD$^{-/-}$ mice (n=5 each) were subjected to hindlimb ischemia. BM cells were obtained from the bilateral femurs by flushing the BM cavity with RPMI 1640 medium. PB and BM cells were suspended in phosphate-buffered saline containing 1% bovine serum albumin and were subjected to flow cytometry. Inflammatory cells in both PB and BM were analyzed using anti-mouse CD45 antibody. EPC-like cells were identified as c-kit$^+$/CD31$^+$ cells. PB and BM cells were incubated with a fluorescein isothiocyanate-conjugated anti-mouse c-kit monoclonal antibody (clone 2B8, Immunotech), followed by phycoerythrin-conjugated anti-mouse CD31 monoclonal antibody (clone MEC13.3, PharMingen). Cells were finally fixed with 1% paraformaldehyde (pH 7.5) in phosphate-buffered saline and analyzed by flow cytometry.

**BM-MNC Differentiation into EPCs.** BM-monoruclear cells (MNCs) were obtained by flushing tibia and femur of WT- and ecSOD$^{-/-}$ mice, and low-density MNCs were then isolated by centrifugation on a Ficoll gradient, as previously described. Fluorescent chemical detection
of endothelial progenitor cells (EPCs) was performed on day 4 after BM-derived MNCs were cultured in fibronectin-coated plate. Fluorescent staining was used to detect binding of fluorescein isothiocyanate-labeled BS lectin (Sigma, St. Louis) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-acLDL; Molecular Probes, Eugene, OR); these are characteristic features of endothelial lineage cells. EPC-like attaching cells were first incubated in medium containing DiI-acLDL (15 µg/ml) for 4 hours at 37°C and then fixed with 1% paraformaldehyde for 10 minutes. After washing, the cells were reacted with BS lectin (10 µg/ml) for 1 hour. After the staining, cells were examined under fluorescence microscopy. Only DiI-acLDL and BS lectin double positive cells were determined to EPCs.

Bone Marrow Transplantation. BM transplantation was performed in lethally irradiated mice (irradiated twice at 5.5 Gy) within 3 hours using BM cells derived from WT and ecSOD−/− mice harvested by flushing femur. Red cells were removed by lysis using ammonium chloride and subsequent washing with PBS. Cells were counted, and 1×10⁶ cells were injected intravenously into the recipient. Then the mice were subjected to hindlimb ischemia and blood flow was measured as described above.

Statistical Analyses. All values were expressed as mean±SE. Blood flow recovery in the ischemic hindlimb (Figure 1A and Figure 4B) was compared between the two groups by two-way repeated measures ANOVA, followed by Bonferroni post hoc analysis. Comparison between two mean values was evaluated by an unpaired Student 2-tailed t test (Figures 1B, 2A, 4C, 5, 6, and 7). Comparison between 3 or more groups was evaluated by one-way ANOVA followed by Bonferroni post hoc analysis (Figures 1C, 1D, 3A, 4A, 8B and Supplemental Figures I and III). Statistical significance was accepted at P<0.05.
**Supplemental Figure legend**

**Supplemental Figure I.** Microcomputed tomography (micro-CT) angiograms demonstrating the impairment of arteriogenesis after hindlimb ischemia in ecSOD−/− mice. A. representative micro-CT images showing collateral formation in WT and ecSOD−/− mice. B. Histomorphometric analysis of vessel connectivity and density (n=3, *p<0.05 vs WT).

**Supplemental Figure II.** Ischemia induced increase in ecSOD expression at Mac3-positive inflammatory cells in hindlimb tissues at 3 days after femoral artery ligation. Photographs are representative of 3 independent experiments.

**Supplemental Figure III.** Ischemia induced apoptosis is enhanced in ecSOD−/− mice. Left panel, representative image of apoptosis in hindlimb tissues in WT and ecSOD−/− mice at 3 days after ischemia, as assessed by terminal dUTP nick-end labeling (TUNEL) assay. White arrow shows TUNEL positive nuclei. Right panel, quantitative analysis of apoptotic cells. Data were expressed as mean TUNEL-positive nuclei numbers per square millimeter. *p<0.05 vs. ischemic tissue from WT mice.
References


Supplemental Figure I

A

Ischemic

Non-ischemic

WT

ecSOD KO

B

vessel connectivity

Connectivity (mm³)

WT     ecSOD KO     WT     ecSOD KO
Non-ischemic     Ischemic

vessel density

Vessel number (1/mm)

WT     ecSOD KO     WT     ecSOD KO
Non-ischemic     Ischemic
Supplemental Figure II

Non-ischemic

ecSOD

Mac3

Ischemic

ecSOD

merge

50 μm
Supplemental Figure III

TUNEL staining

WT

ecSOD KO

Non-ischemic

Ischemic

TUNEL positive Nuclei/mm²

Non-ischemic

Ischemic

WT

coSOD KO

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