Role of gp91phox (Nox2)-Containing NAD(P)H Oxidase in Angiogenesis in Response to Hindlimb Ischemia

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Background—Neovascularization is potentially important for the treatment of ischemic heart and limb disease. We reported that reactive oxygen species (ROS) derived from gp91phox (Nox2)-containing NAD(P)H oxidase are involved in angiogenesis in mouse sponge models as well as in vascular endothelial growth factor (VEGF) signaling in cultured endothelial cells. The role of gp91phox-derived ROS in neovascularization in response to tissue ischemia is unknown, however.

Methods and Results—Here, we show that neovascularization in the ischemic hindlimb is significantly impaired in gp91phox−/− mice as compared with wild-type (WT) mice as evaluated by laser Doppler flow, capillary density, and microsphere measurements. In WT mice, inflammatory cell infiltration in the ischemic hindlimb was maximal at 3 days, whereas capillary formation was prominent at 7 days when inflammatory cells were no longer detectable. Increased O2− production and gp91phox expression were present at both time points. The dihydroethidium staining of ischemic tissues indicates that O2− is mainly produced from inflammatory cells at 3 days and from neovasculature at 7 days after operation. Relative to WT mice, ischemia-induced ROS production in gp91phox−/− mice at both 3 and 7 days was diminished, whereas VEGF expression was enhanced and the inflammatory response was unchanged. Infusion of the antioxidant ebselen into WT mice also significantly blocked the increase in blood flow recovery and capillary density after ischemia.

Conclusions—gp91 phox-derived ROS play an important role in mediating neovascularization in response to tissue ischemia. NAD(P)H oxidases and their products are potential therapeutic targets for regulating angiogenesis in vivo. (Circulation. 2005;111:2347-2355.)

Key Words: NAD(P)H oxidase ■ reactive oxygen species ■ angiogenesis ■ ischemia ■ vascular endothelial growth factor

Neovascularization stimulated by tissue ischemia is important to the preservation of tissue integrity and function. New blood vessels grow postnatally via angiogenesis (capillary sprouting from the preexisting blood vessels), arteriogenesis (in situ growth of preexisting arteriolar connections into true collateral arteries), and vasculogenesis.1 Inflammation is an important early key process for ischemia-induced angiogenesis and arteriogenesis.2,3 Angiogenesis requires degradation of extracellular matrix, endothelial cell (EC) proliferation and migration, and organization into tubes with lumen formation.4 Vascular endothelial growth factor (VEGF) is an important endogenous regulator of angiogenesis in tissue ischemia.5 In ECs, VEGF binds to 2 tyrosine kinase receptors, VEGF receptor (VEGFR) 1 (Flt-1), and VEGFR2 (KDR/Flik1). The mitogenic and chemotactic effects of VEGF in ECs are mediated mainly through VEGFR2.6 VEGF also is involved in mobilization of endothelial progenitor cells (EPCs) from the bone marrow, which contributes to new vessel formation in ischemic tissues.7 Reactive oxygen species (ROS) such as superoxide anion (O2−) and hydrogen peroxide (H2O2) are involved in the signaling pathways that mediate many stress and growth responses,8 including angiogenesis.9 Although ROS in high concentrations are toxic,10 low levels of ROS produced during cardiac ischemic preconditioning serve as intracellular signals stimulating counterregulatory mechanisms that prevent tissue injury and promote angiogenesis.11,12 Indeed, ROS produced during myocardial ischemia/reperfusion are involved in neovascularization.14 In ECs, NAD(P)H oxidase is a major source of ROS,15 which are required for EC proliferation and migration.16 ECs express NAD(P)H oxidase subunits that are identical to those found in phagocytes. These include the membrane-bound flavocytochrome b558, composed of gp91phox (now known as Nox2) and p22phox and the

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cytosolic components p47(phox) and p67(phox). On stimulation, a multimeric protein complex, also including the small guanosine triphosphatase Rac1, is formed that is associated with the production of O$_2^−$.

We showed that VEGF stimulates an increase in ROS generation via activation of gp91(phox), containing NAD(P)H oxidase in ECs. Thus, ROS are critical downstream mediators of VEGF-mediated signaling linked to angiogenic responses in ECs. Using a sponge implant model, we showed that VEGF-induced new blood vessel formation is impaired in gp91(phox)$^{-/-}$ mice; however, the role of ROS and NAD(P)H oxidase in neovascularization that occurs naturally in response to hindlimb ischemia is undefined.

In the present study, we demonstrate that neovascularization in ischemic hindlimbs of mice is associated with a robust increase in ROS as well as upregulation of gp91(phox) protein. We also found that infiltrated inflammatory cells and activated or newly formed ECs are the major source of ROS at 3 and 7 days after ischemia, respectively. In gp91(phox)$^{-/-}$ mice, ischemia-induced neovascularization as well as an increase in ROS production at both time points were significantly impaired. These findings suggest that ROS derived from gp91(phox)$^{-/-}$-containing NAD(P)H oxidase are important mediators of neovascularization triggered by tissue ischemia. NAD(P)H oxides and their products may be attractive therapeutic targets for modulating angiogenesis in vivo.

Methods

Mouse Ischemic Hindlimb Model

Female gp91phox$^{-/-}$ mice and wild-type (WT) C57BL/6J mice (8 to 9 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were subjected to unilateral hindlimb surgery under anesthesia with intraperitoneal administration of ketamine (87 mg/kg). Mice were 8 to 9 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, Maine). 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 and vein were exposed, ligated proximally and distally with 5-0 silk ligatures, and excised. In some experiments, the antioxidant ebselen (Cayman Chemical Co; 50% vol/vol in DMSO, 10 mg · kg$^{-1}$ · d$^{-1}$) or saline (50% vol/vol in DMSO) was chronically infused by osmotic minipump (Durect) from 1 day before operation. The Animal Care and Use Committee of the Emory University School of Medicine approved study protocols.

Laser Doppler Blood Flow Analysis

We measured hindlimb blood flow using a laser Doppler blood flow (LDFB) analyzer (Lisca AB) as described previously. Mice were anesthetized and placed on a heating plate at 37°C for 15 minutes to minimize temperature variation. Before surgery and on postoperative days 0, 3, and 7, LDFB analysis was performed on legs and feet. Blood flow was displayed as changes in the laser frequency, representing the number of infiltrated CD45-positive cells, macrophages and lectin-positive capillary cells were counted in 5 randomly selected high-power fields (magnification ×400) and data from 3 fields of the 5 samples were averaged.

Blood Flow Measurement by Microsphere

Mice were euthanized, and fluorescent microspheres (15-μm diameter, 5 × 10$^{10}$ 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, MIL- LIPORE). The fluorescent dye was extracted with xylene, and fluorescence was measured with a CytoFluor 3000 plate reader (Applied Biosystems) and normalized by muscle weight.

Chemiluminescence Estimates of Superoxide Produced by Hindlimb Tissues

Superoxide production by ischemic and nonischemic hindlimb tissues was measured with 5 μmol/L lucigenin-enhanced chemiluminescence, as previously described. 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$_2^−$.

Histological Analysis and In Situ Localization of ROS

Seven-micrometer-thick sections prepared from paraffin-embedded tissue of the ischemic and control hindlimbs were used for histological analysis by hematoxylin-and-eosin (H&E) staining. For in situ localization of ROS, we incubated frozen sections (30 μm) of ischemic and nonischemic tissues with fluorophores sensitive to O$_2^−$, dihydroethydium (DHE, 10 μmol/L, Sigma), or hydrogen peroxide (H$_2$O$_2$, 5- and 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (DCF-DA; 10 μmol/L, Molecular Probes). DHE specifically reacts with intracellular O$_2^−$ and is converted to the red fluorescent compound ethidium, which then binds irreversibly to double-stranded DNA and appears as punctate nuclear staining. The specificity of DHE and DCF signals for O$_2^−$ and H$_2$O$_2$ detection was confirmed by preincubation with polyethylene glycol-superoxide dismutase (PEG-SOD; 500 U/mL, Sigma) and PEG-catalase (350 U/mL, Sigma), respectively. To determine which cells produce ROS robustly, serial frozen OCT-embedded tissue sections (7 μm) were stained for 1 hour at room temperature with anti–mouse CD45 antibody, which detects inflammatory leukocytes (PharMingen), with anti-mouse mac3 antibody, which detects macrophage specifically (Accurate Chemical & Scientific Corp), or with G simplicifolia lectin (Vector Laboratories), which stains ECs or anti-gp91phox antibody (Santa Cruz). The slides were then incubated for 1 hour at room temperature with a Rhodamine Red-X–conjugated anti-rabbit secondary antibody or fluorescein isothiocyanate–conjugated anti-rat antibody (Jackson ImmunoResearch Laboratories). For quantification, the number of infiltrated CD45-positive cells, macrophages and lectin-positive capillary cells were counted in 5 randomly selected high-power fields (magnification ×400) with 3 independent samples.

Western Blotting

Ischemic and control hindlimbs were lysed in lysis buffer, pH 7.4 (in mmol/L: 50 HEPES, 5 EDTA, 50 NaCl), 1% Triton X-100, 60 mmol/L n-octylglucoside, protease inhibitors (10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin), and phosphatase inhibitors (in mmol/L: 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate). Cell lysates were used for immunoblotting with antibodies for VEGF (Santa Cruz).

Statistical Analyses

All values were expressed as mean ± SE. The significance of the differences between groups was evaluated by a Student paired 2-tailed t test. The values in >3 groups were tested by 1-way ANOVA.
Results

Ischemia-Induced Neovascularization Is Impaired in gp91phox−/− Mice

All mice survived after induction of unilateral hindlimb ischemia and appeared to be healthy during the follow-up period. To determine the role of gp91phox in ischemia-induced neovascularization, we measured blood flow recovery using LDBF analysis in ischemic and nonischemic limbs after femoral ligation in WT and gp91phox−/− mice. Figure 1A shows that in WT mice, hindlimb blood flow was markedly decreased immediately after surgery, was partially restored at day 3, and had recovered to the level of that of the nonischemic tissues at 7 days after operation. In gp91phox−/− mice the flow recovery was delayed, and the LDBF ratio at 7 days after ischemia was significantly less than that in WT mice.

Neovascularization induced by tissue ischemia is mediated through both angiogenesis and arteriogenesis.1 We examined the role of gp91phox in ischemia-induced angiogenesis by measuring capillary density in ischemic and nonischemic tissues. Figure 1B shows representative photomicrographs stained with endothelium-specific G simplicifolia lectin.19 We used lectin instead of CD31 to detect ECs because of the high staining efficiency of lectin as opposed to CD31 staining in our system. Quantitative analysis revealed that the capillary density in ischemic hindlimbs at 7 days after ischemia was significantly reduced in gp91phox−/− mice as compared with WT mice.

To determine the role of gp91phox in ischemia-induced arteriogenesis, we used microspheres of 15 μm diameter to analyze the formation of conductance collateral vessels.23,24 As shown in Figure 1C, the recovery of collateral blood flow at 7 days after hindlimb ischemia was significantly reduced in gp91phox−/− mice as compared with WT mice. Taken together, these results suggest that both angiogenesis and arteriogenesis are regulated by gp91phox protein expression.

ROS Production Via gp91phox-Containing NAD(P)H Oxidase in Response to Hindlimb Ischemia

We examined whether hindlimb ischemia stimulates O2− production by using lucigenin assays. As shown in Figure 2, there was a significant increase in O2− production in ischemic tissues at 3 and 7 days after operation in WT mice. Ischemia-induced O2− production at each time point was significantly diminished in gp91phox−/− mice, suggesting that gp91phox-containing NAD(P)H oxidase is a major source of ROS produced in both the early and the later phases of neovascularization after hindlimb ischemia.

Identification of O2−-Producing Cells

The gp91phox-containing NAD(P)H oxidases are expressed in both phagocytes and ECs.15,25 Figure 3A shows H&E staining of ischemic and nonischemic tissues at 3 days after femoral ligation in WT mice and demonstrates a marked increase in infiltration of inflammatory cells in ischemic hindlimb as compared with nonischemic tissues, as has been reported by others.3 To determine whether O2− is produced from inflammatory cells on day 3, we performed DHE staining, which is specific for O2−,21 and immunofluorescence staining of serial sections for CD45-positive leukocytes and macrophages in ischemic tissues. As shown in Figure 3B, the increase in O2− was detected in ischemic tissues as compared with nonischemic tissues at 3 days after operation, and the pattern of staining for CD45-positive cells and macrophages was similar to that obtained with DHE. In contrast, as shown in Figure 3C, H&E staining at day 7 shows that capillary-like structures appeared in increased density in ischemic tissues, which is consistent with the observed increase in lectin staining, whereas inflammatory cells are not present at this time point. We therefore hypothesized that augmented ROS production on day 7 could be related to enhanced generation by the activated ECs. As shown in Figure 3D, there was an increase in DHE staining in ischemic tissues on day 7, which was almost completely abolished by coinoculation with 500 U/mL PEG-SOD, providing specific evidence for the presence of increased O2−. Figure 3E shows that DHE staining appears to be associated with capillary-like lectin-positive ECs in ischemic tissues. Of note, there were few CD45- and macrophage-positive cells at 7 days (not shown). These data suggest that inflammatory cells and neovascular ECs are responsible for ROS production in the early (day 3) and the later (day 7) phases, respectively, after the induction of hindlimb ischemia.

gp91phox Protein Expression in Response to Hindlimb Ischemia

To gain insight into the mechanisms by which ischemia increases ROS production, we examined, using immunocytochemistry, the expression of gp91phox in ischemic and nonischemic tissues at 3 and 7 days after femoral artery ligation in WT mice. As shown in Figure 4, gp91phox was dramatically increased in ischemic tissues at both 3 and 7 days after operation. Double staining after ischemic injury for gp91phox and CD45 or lectin shows that gp91phox protein appears to be prominently expressed by infiltrating inflammatory leukocytes at 3 days and by newly formed capillaries at 7 days. Using Western blot analysis, we found that p22phox, p47phox, and p67phox protein expression is also upregulated in ischemic tissues at both 3 and 7 days after operation (data not shown).

Infiltration of Inflammatory Cells and Induction of VEGF Expression Are Not Decreased in gp91phox−/− Mice

We examined whether the impairment of angiogenesis and ROS production in gp91phox−/− mice is due to the reduction of infiltration of inflammatory cells that release ROS and angiogenic factors. As shown in Figure 5A, there was no significant difference in the number of infiltrated CD45-positive leukocytes and macrophages in ischemic tissues between WT and gp91phox−/− mice at 3 or 7 days. Furthermore, because VEGF is a major mediator of ischemia-induced angiogenesis4 and stimulates ROS production by ECs,17,18,26 we examined the expression of VEGF in the ischemic tissues of WT and gp91phox−/− mice. Figure 5B
shows that there was a significant increase in VEGF expression at 3 and 7 days in WT mice and an even more robust enhancement of expression in gp91phox/H11002/H11002/H11002 mice. Taken together, these results suggest that impairment of angiogenesis in gp91phox/H11002/H11002/H11002 mice is not due to the decrease of inflammatory cells or VEGF expression but to the inhibition of their downstream ROS production in ischemic tissues.

**Ebselen Inhibits Ischemia-Induced Angiogenesis**

To gain further insight into the role of increased ROS production in ischemia-induced angiogenesis, we examined in WT mice the effect of the glutathione peroxidase mimetic ebselen, which scavenges H2O2. Infusion of ebselen markedly reduced at day 7 the ischemia-induced increase in the LDBF ratio (Figure 6A) and capillary density (Figure 6B). Ischemia-induced increase in DCF fluorescence at day 7 was markedly inhibited by ebselen and by PEG-catalase (Figure 6C) but was only slightly reduced by PEG-SOD (data not shown). These results provide additional support for the notion that ROS, predominantly H2O2, are important for neovascularization induced by tissue ischemia.

**Discussion**

New blood vessel formation in response to ischemia is an important adaptive response for preserving tissue integrity and is regulated by hypoxia and inflammation. The present study provides direct evidence that hindlimb ischemia in mice induces a significant increase in ROS production in the ischemic area, not only as an early response to injury but also at 7 days after femoral ligation (Figures 2 and 3). Histological analysis reveals that inflammatory cell infiltration into the ischemic hindlimb is observed maximally at 3 days (Figure 3) when blood flow is significantly reduced (Figure 1), whereas and gp91phox/H11002/H11002/H11002 mice at 7 days after ischemia. Capillary density was expressed as the number of capillaries per high-power field (magnification ×400, per square millimeter; n=5, *P<0.05 vs WT). C, Quantitative analysis of collateral blood flow of hindlimbs using 15-μm-diameter fluorescent microspheres. Data are shown as ischemic/nonischemic fluorescence ratio (n=8, *P<0.05 vs WT).
new capillary formation is prominent at 7 days when inflammatory cells are no longer detectable (Figure 3) and blood flow has almost completely recovered (Figure 1). Of note, the extent of ROS production on day 7 is much higher than that on day 3 (Figure 2), suggesting that the increase in ROS levels is related to neovascularization. The early recruitment of inflammatory cells to the ischemic sites has been shown to be important for the subsequent development of angiogenesis induced by hindlimb ischemia. DHE staining of ischemic tissues reveals that the source of $\text{O}_2^-$ is mainly from infiltrated inflammatory cells at 3 days and from lectin-positive ECs at 7 days after ischemia (Figure 3). This method has been used to localize $\text{O}_2^-$ production within the vessel wall. These observations indicate that hindlimb ischemia stimulates increase in ROS production initially from inflammatory cells and subsequently from the neovasculature.

NAD(P)H oxidase is a major enzymatic source of ROS produced in both inflammatory cells and ECs. In neutrophils, the full electron transport function of the respiratory burst NAD(P)H oxidase resides in gp91phox, which interacts with p22phox to form cytochrome b558. We and others have shown that gp91phox is a critical component for $\text{O}_2^-$-generating NAD(P)H oxidase in ECs. The present study demonstrates that expression of the gp91phox protein in ischemic tissues is increased concomitantly with the elevation in ROS levels at both 3 and 7 days after occlusion (Figure 4). Furthermore, other NAD(P)H oxidase components such as p22phox, p47phox, and p67phox proteins are also upregulated in ischemic hindlimbs. We also show that ischemia-induced increase in ROS production at both time points is almost completely abolished in gp91phox−/− mice (Figure 2). These data suggest that gp91phox-containing NAD(P)H oxidase is a major source of ROS produced from inflammatory cells in the early phase and from activated or newly formed ECs in the later phase during ischemia-induced angiogenesis.
Neovascularization in the adult occurs through angiogenesis (capillary growth) and arteriogenesis (collateral growth).\textsuperscript{1} In the present study, we evaluated angiogenesis by measuring capillary density with lectin staining and arteriogenesis by the formation of large conductance of collateral vessels using microspheres with 15-μm diameters, which do not pass through small capillaries.\textsuperscript{24} Using these techniques in addition to the laser Doppler perfusion imaging, we demonstrated that ischemia-induced blood flow recovery, collateral blood flow, and an increase in capillary density were significantly inhibited in gp91\textsuperscript{phox−/−} mice (Figure 1). Furthermore, infusion of ebselen, which scavenges H\textsubscript{2}O\textsubscript{2}, significantly inhibits ischemia-induced increase in blood flow restoration, in capillary density, and in H\textsubscript{2}O\textsubscript{2} generation in WT mice. These data strongly suggest that ROS derived from gp91\textsuperscript{phox}-containing NAD(P)H oxidase play an important role in ischemia-induced new blood vessel formation. It should be noted that ebselen also scavenges peroxynitrite,\textsuperscript{30} which is produced through the reaction of O\textsubscript{2}− with nitric oxide. Given that a role of nitric oxide has been implicated in neovascularization\textsuperscript{31,32} and O\textsubscript{2}− is generated in response to tissue ischemia (present study), it is possible that peroxynitrite also may be involved in ischemia-induced angiogenesis. In the present study, ischemia-induced increase in DCF fluorescence in ischemic tissues is markedly inhibited by PEG-catalase (Figure 6) but is only modestly reduced by PEG-SOD. Thus, postischemic increase in DCF fluorescence appears to reflect enhanced production of H\textsubscript{2}O\textsubscript{2}, but a contribution of peroxynitrite cannot be excluded. Our results are consistent with the possibility that H\textsubscript{2}O\textsubscript{2} derived from the gp91\textsuperscript{phox}-containing NAD(P)H oxidase at least in part plays an important role in neovascularization in response to tissue ischemia. Because circulating EPCs also contribute to postnatal neovascularization,\textsuperscript{33,34} we cannot exclude the possibility that gp91\textsuperscript{phox}-derived ROS also may be involved in mobilization or incorporation of EPCs to the neoangiogenic sites.

Other reports implicate a role for ROS in neovascularization in experimental models. Hypoxia/reoxygenation in hearts produces ROS, which in turn is associated with myocardial angiogenesis\textsuperscript{13} and coronary collateral development.\textsuperscript{35} Correlation between ROS production and neovascularization has been demonstrated in the eyes of diabetic rats,\textsuperscript{36,37} the neointima of balloon-injured rat arteries,\textsuperscript{38} and the response to carotid artery ligation in mice overexpressing p22\textsuperscript{phox}.\textsuperscript{22}

![Figure 4](http://circ.ahajournals.org/) Induction of gp91\textsuperscript{phox} protein expression in response to hindlimb ischemia. Ischemia-induced increase in gp91\textsuperscript{phox} protein expression in nonischemic and ischemic hindlimb tissues in WT mice at 3 and 7 days after femoral artery ligation. Photographs are representative of 3 independent experiments.

![Figure 5](http://circ.ahajournals.org/) Ischemia-induced infiltration of inflammatory cells and induction of VEGF expression are not decreased in gp91\textsuperscript{phox−/−} mice. Quantitative analysis of the number of infiltrated CD45-positive inflammatory leukocytes and macrophages (A) and VEGF protein expression (B) in hindlimb ischemic tissues in WT and gp91\textsuperscript{phox−/−} mice at 0 (for B), 3, and 7 days after femoral artery ligation (n=3, NS; not significant, *P<0.05 and †P<0.01 vs WT mice at day 0).
tioxidant treatment inhibits neovascularization in the mouse cornea angiogenesis model and tumor angiogenesis model in vivo. Overexpression of extracellular SOD, which scavenges $O_2^-$, reduces tumor angiogenesis. Moreover, we have shown that VEGF-induced new vessel formation in a sponge implant model is impaired in gp91phox mice. Our study extends and amplifies these data by identifying the gp91phox-containing NAD(P)H oxidase as a major source of ROS involved in adaptive angiogenesis in response to tissue ischemia.

It has been shown that infiltrating inflammatory cells release cytokines and angiogenic factors, including VEGF, and thus promote collateral development and angiogenesis in hindlimb ischemia. In the present study, we demonstrate that the absence of gp91phox did not inhibit recruitment of inflammatory cells (assessed at day 3) or the increased expression of VEGF at 3 or 7 days after operation (Figure 5). These observations are in line with previous reports that gp91phox-knockout mice show normal inflammatory responses to bacteria and to hindlimb unloading/reloading, which induces muscle inflammation. Thus, gp91phox-derived ROS may be important in mediating signaling responses in the neovasculature itself. This notion is consistent with our previous report that stimulation of endothelial ROS production from gp91phox-containing NAD(P)H oxidase is necessary for VEGF-induced autophosphorylation and activation of VEGFR2, as well as cell proliferation and migration in ECs. Moreover, using laser Doppler flow analysis, we found that ischemia-induced increase in blood flow recovery in the later phase is inhibited in transgenic mice overexpressing catalase in the vascular endothelium (M. Ushio-Fukai, PhD, unpublished data, April 2005). These findings emphasize an impor-

Figure 6. Scavenging $H_2O_2$ inhibits angiogenesis in response to hindlimb ischemia. A, Quantitative analysis (top) and representative images (bottom) of the ischemic/nonischemic LDBF ratio in WT mice treated with saline (closed circle) or antioxidant ebselen (open circle, 10 mg · kg$^{-1}$ · d$^{-1}$) before surgery and on postoperative days 0, 3, and 7 (n=7, *$P<0.05$ vs saline). Arrows indicate impairment of blood flow recovery in ebselen-treated WT mice at 7 days after ischemia. B, Representative pictures (left) and quantification (right) of G simplicifolia lectin staining (green) to detect capillary density in ischemic and nonischemic tissues in WT mice treated with saline or ebselen at 7 days (n=5, *$P<0.05$ vs saline). C, DCF staining in ischemic tissues in WT mice treated with saline or ebselen or PEG-catalase (350 U/mL) at 7 days after operation, suggesting that $H_2O_2$ is predominantly produced after ischemia. Photographs are representative of 3 independent experiments.
tant role for EC-derived ROS in new blood vessel formation triggered by tissue ischemia.

Neither the infiltration of inflammatory cells nor their production of VEGF and possibly other angiogenic factors is affected after ischemia, whereas ROS production from these cells on day 3 is markedly diminished in gp91phox⁻/− mice. Previous studies show that hindlimb ischemia–induced early recruitment of inflammatory cells is temporally associated with the activation of matrix metalloproteinases (MMP)-2 and -9, which also are involved in postnatal revascularization.⁴⁶ Given that ROS activates MMP-9 in isolated macrophages⁴⁷ and macrophage-derived MMP-9 plays an important role in ischemia-induced angiogenesis,⁹ ROS produced from inflammatory cells may be required for the subsequent activation of ECs through regulating MMP activity. Definitive answers about the relative roles of leukocyte versus EC gp91phox in ischemia-induced neovascularization will require further investigation. Our current data, however, suggest that gp91phox-derived ROS exert a major influence on events downstream of VEGF generation, most likely, in signaling pathways in ECs.

In summary, the present study provides compelling evidence that ROS derived from gp91phox-containing NAD(P)H oxidase play a critical role in ischemia-induced neovascularization. We found that gp91phox-derived ROS are produced by mobilizing bone marrow–derived endothelial progenitor cells. EMBO J. 1999;18:3964–3972.


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