Nox2-Containing NADPH Oxidase Deficiency Confers Protection From Hindlimb Ischemia in Conditions of Increased Oxidative Stress

Paola Haddad, Sylvie Dussault, Jessika Groleau, Julie Turgeon, Sophie-Elise Michaud, Catherine Ménard, Gemma Perez, Fritz Maingrette, Alain Rivard

Objective—Because Nox2-containing NADPH oxidase is a major source of ROS in the vasculature, we investigated its potential role for the modulation of ischemia-induced neovascularization in conditions of increased oxidative stress.

Methods and Results—To mimic a clinical situation of increased oxidative stress, mice were exposed to cigarette smoke before and after the surgical induction of hindlimb ischemia. Nox2 expression and oxidative stress in ischemic tissues were significantly increased in wild-type mice, but not in mice deficient for the Nox2-containing NADPH oxidase (Nox2<sup>−/−</sup>). Nox2<sup>−/−</sup> mice demonstrated faster blood flow recovery, increased capillary density in ischemic muscles, and improved endothelial progenitor cell functional activities compared to Nox2<sup>+/+</sup> mice. In addition, Nox2 deficiency was associated with increased antioxidant and nitrite concentrations in plasma, together with a preserved expression of eNOS in ischemic tissues. In vitro, Nox2<sup>−/−</sup> endothelial cells exhibit resistance against superoxide induction and improved VEGF-dependent angiogenic activities compared to Nox2<sup>+/+</sup> endothelial cells. Importantly, the beneficial effects of Nox2 deficiency on neovascularization in vitro and in vivo were lost after treatment with the NO inhibitor L-NAME.

Conclusions—Nox2-containing NADPH oxidase deficiency protects against ischemia in conditions of increased oxidative stress. The mechanism involves improved neovascularization through a reduction of ROS formation, preserved activation of the VEGF/NO angiogenic pathway, and improved functional activities of endothelial progenitor cells. (Arterioscler Thromb Vase Biol. 2009;29:1522-1528.)

Key Words: NADPH oxidase ▪ neovascularization ▪ cigarette smoking ▪ nitric oxide ▪ endothelial progenitor cells

The capacity of the organism to develop new blood vessels (neovascularization) constitutes an important adaptive mechanism against ischemia. In the adult, neovascularization is the result of 3 different processes: angiogenesis (capillary sprouting from preexisting blood vessels), collateral growth, and vasculogenesis (development of neovessels from endothelial progenitor cells [EPCs]). Vascular endothelial growth factor (VEGF), an endothelial cell specific mitogen, has been shown to be a critical limiting factor for the induction of angiogenesis. The angiogenic effects of VEGF have been associated with the production of nitric oxide (NO). NO is an essential mediator of endothelial cell migration and VEGF-induced angiogenesis. Moreover, NO was recently shown to be involved in the mobilization of EPCs from the bone marrow.

Interestingly, the same risk factors that promote the development of atherosclerotic vascular diseases are also associated with reduced neovascularization in response to ischemia. A common feature of all these cardiovascular risk factors is increased production of reactive oxygen species (ROS) and reduced NO bioactivity. Low levels of ROS including superoxide and hydrogen peroxide can serve as intracellular signals in response to ischemia, stimulating mechanisms that prevent tissue injury and promote angiogenesis. However, excessive production of ROS leads to cellular toxicity and has been associated with impaired angiogenesis in different models. A major source of ROS in the vessel wall is the family of membrane-associated NADPH oxidases. NADPH oxidase consists of a membrane-localized cytochrome b558 composed of gp91<sub>phox</sub> (or Nox2) and p22<sub>phox</sub> and the cytosolic components p47<sub>phox</sub> and p67<sub>phox</sub>. On stimulation, a multimeric protein complex (involving also Rac1) is formed, leading to the production of superoxide. Recent evidence indicates that Nox2-containing NADPH oxidase is a major source of superoxide and ROS in endothelial cells. It has been suggested that Nox2-containing NADPH oxidase-derived ROS play a role in VEGF-induced endothelial cell signaling and angiogenesis. Similarly, Nox2-deficient mice have been shown to present impaired angiogenesis in response to hindlimb ischemia. These studies however were performed in young and healthy animals, a situation that does not reflect the state of increased...
oxidative stress encountered in patients with advanced ischemic cardiovascular diseases. The hypothesis of the current study was that in a clinical situation of increased oxidative stress, excessive ROS production by Nox2-containing NADPH oxidase would contribute to impair ischemia-induced neovascularization.

Patients with cardiovascular diseases often present several risk factors that are associated with increased oxidative stress including aging, hypercholesterolemia, hypertension, and diabetes. However, in developed countries, cigarette smoking is recognized as the leading modifiable risk factor associated with ischemic cardiovascular diseases and premature death. We have previously shown that cigarette smoking impairs ischemia-induced neovascularization. We also recently demonstrated that cigarette smoke exposure impairs VEGF-dependent angiogenesis, and that this is at least partly attributable to the generation of ROS and the inhibition of NO bioactivity. In the current study, to mimic a clinical situation of increased oxidative stress, animals were exposed to cigarette smoke before and after the surgical induction of hindlimb ischemia. Our aim was to investigate the role of Nox2-containing NADPH oxidase for the modulation of ischemia-induced neovascularization.

Methods
For expanded Methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Murine Ischemic Hindlimb Model
Six- to 8-week-old Nox2/−/− mice on a C57BL/6 background were purchased from Jackson Laboratory (Jackson Mice, Bar Harbor, Me). After 2 weeks of cigarette smoke exposure, unilateral hindlimb ischemia was surgically induced. In some experiments, mice were treated with the NO inhibitor NG-nitro-L-arginine methyl ester (L-NAME 1 mg/mL of drinking water). Hindlimb perfusion was measured with a Laser Doppler Perfusion Imager (LDPI) system (Moor Instruments Ltd.).

Cigarette Smoke Exposure
Mice were started on cigarette exposure (1 cigarette, twice a day) via a smoking machine described previously. This exposure is associated with carboxyhemoglobin blood levels that are similar to those of chronic smokers (8% to 14%).

Immunohistochemistry
Identification of endothelial cells in ischemic muscles was performed by immunostaining for platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) with a rat monoclonal antibody directed against mouse CD31 (Pharmigen). To evaluate local oxidative stress levels in ischemic muscles, nitrotyrosine immunostaining (Upstate) and dihydroethidium staining (DHE, Molecular Probes) were used.

FACS Analysis of Peripheral Blood EPCs
The percentages of EPCs contained in the total viable cell population derived from the peripheral blood was measured by FACS (FACS-Calibur flow cytometer) using the following fluorescence-coupled endothelial cell markers: CD34-FITC and Flk1-PE (eBioscience).

Aortic Endothelial Cells and EPC Isolation
Endothelial cells from the thoracic aorta were isolated by an explant technique. EPCs were isolated from the femora and tibiae by flushing the bone marrow cavities using culture medium as previously described. Bone marrow EPCs were characterized as adherent cells that were positive for both Dil-acLDL uptake and lectin binding.

Plasma Antioxidant Potential and NO Release
Antioxidant concentrations in mice plasma samples were determined using the total antioxidant status assay kit (CayBiochem Biochemicals). NO production was determined indirectly by measuring the concentration of the stable end products nitrate and nitrite using a commercial kit (R&D systems Inc) based on the Griess reaction.

Western Blot Analysis
The following antibodies were used: 1:500 gp91 (Transduction Laboratories), 1:200 p47 and p67 (Santa Cruz Biotechnology), 1:1000 eNOS (Cell Signaling), and 1:200 α-tubulin (Santa Cruz Biotechnology). Results are expressed as density values normalized to α-tubulin.

Preparation of Cigarette Smoke Extracts
Cigarette smoke extracts (CSE) were prepared as previously described. CSE was applied to mouse aortic endothelial cells or EPCs in culture for 16 hours within 30 minutes of preparation to obtain a final concentration of 10%. This concentration corresponds approximately to exposure associated with smoking 1.5 pack per day.

Cell Migration and Capillary-Like Tube Formation
Cell migration was assessed using a modified Boyden chamber assay. Capillary-like tube formation was assessed using a matrigel assay.

Detection of Intracellular Superoxide and NO
Intracellular generation of O2− and NO were visualized with the fluoroprobes dihydroethidium (DHE, Molecular Probes) and diaminofluorescein-2-diacetate (DAF-2DA, Cell Technology), respectively.

Statistical Analysis
All results are expressed as mean±SEM. Statistical significance was evaluated by ANOVA. A value of P<0.05 was interpreted to denote statistical significance.

Results
NADPH Oxidase Subunit Protein Expression and ROS Levels in Ischemic Tissues
We investigated the effect of cigarette smoke exposure on the expression of NADPH oxidase subunits in ischemic muscles harvested at day 6 after surgery (supplemental Figure 1). We found that Nox2, p22, and p47 were significantly increased in mice exposed to cigarette smoke. In contrast, p67 expression was not modified by cigarette smoke exposure. We also confirmed that Nox2/−/− mice had a selective ablation of the Nox2 catalytic subunit in ischemic muscles, but did express the other subunits of NADPH oxidase. Nitrotyrosine and DHE immunostaining demonstrated that the level of ROS is significantly increased in ischemic tissues of wild-type mice exposed to cigarette smoke (Figure 1A through 1D). However, Nox2/−/− mice were protected against the increase of oxidative stress in ischemic tissues.

Nox2 Deficiency Improves Ischemia-Induced Neovascularization
Hindlimb perfusion was evaluated postoperatively by serial LDPI studies (Figure 2A and 2C). In wild-type mice, ciga-
Cigarette smoke exposure was associated with a significant impairment of blood flow recuperation at day 7 (Doppler flow ratio (DFR) 0.57 ± 0.04 versus 0.67 ± 0.03; P < 0.05) and day 14 (0.61 ± 0.02 versus 0.76 ± 0.02; P < 0.01) after surgery. In basal conditions, Nox2<sup>−/−</sup> mice exhibited improved blood flow recuperation compared to wild-type at day 7 (0.80 ± 0.05 versus 0.67 ± 0.03; P < 0.05) and at day 14 (0.93 ± 0.05 versus 0.76 ± 0.02; P < 0.01). Moreover, Nox2<sup>−/−</sup> mice were protected against cigarette smoke–induced impairment of blood flow recuperation. Therefore, the ultimate blood flow recuperation in mice exposed to cigarette smoke was dramatically improved in Nox2<sup>−/−</sup> compared to wild-type mice at day 7 (0.80 ± 0.04 versus 0.57 ± 0.04; P < 0.001) and at day 14 (0.89 ± 0.04 versus 0.61 ± 0.02; P < 0.001). Similar findings were observed at the microvascular level (Figure 2B and 2D). In wild-type mice, cigarette smoke exposure was associated with a significant decrease of capillary density in ischemic muscles compared to wild-type mice at day 7 (583 ± 36 versus 418 ± 19 capillaries/mm<sup>2</sup>; P < 0.001). In basal conditions, Nox2<sup>−/−</sup> mice exhibited increased capillary density in ischemic muscles compared to wild-type mice (583 ± 36 versus 418 ± 19 capillaries/mm<sup>2</sup>; P < 0.001). Moreover, capillary density was not significantly reduced by cigarette smoke exposure in Nox2<sup>−/−</sup> mice. Therefore, the capillary density in ischemic muscles of mice exposed to cigarette smoke was dramatically increased in Nox2<sup>−/−</sup> compared to wild-type mice (535 ± 21 versus 280 ± 16 capillaries/mm<sup>2</sup>; P < 0.001).

Nox2 Deficiency Is Associated With Improved EPC Functional Activity
It has been demonstrated that circulating EPCs in adults can home to ischemic tissues and contribute to the formation of new blood vessels. Accordingly, here we determined the effect of Nox2 deficiency on the number and the functional activity of EPCs. We found that the number of peripheral blood EPCs was similar in mice exposed or not to cigarette smoke, and in Nox2<sup>−/−</sup> compared to wild-type mice (Figure 3A). However, we demonstrate that VEGF-induced migratory activity is significantly reduced in wild-type EPCs exposed to 10% CSE (Figure 3B). This is associated with a cigarette smoke–dependant increase of oxidative stress level (Figure 3C and 3E) and a modest but significant reduction of NO production (Figure 3D and 3F). Importantly, Nox2 deficiency was associated with reduced oxidative stress level, improved NO production, and rescued migratory activity in EPCs exposed to CSE (Figure 3B through 3F).

Nox2 Deficiency Reduces Oxidative Stress and Restores VEGF-Dependent Angiogenic Activities in Mature Endothelial Cells
Superoxide generation was measured using DHE staining in endothelial cells isolated from the aortas of Nox2<sup>−/−</sup> and wild-type mice. We found that the exposure to 10% CSE was...
associated with a significant increase of superoxide anion generation in wild-type endothelial cells (Figure 4A and 4C). This increased oxidative stress correlated with reduced VEGF-induced tube formation (Figure 4B and 4D) and cellular migration (Figure 4E). We also demonstrated that the effects of CSE are NADPH oxidase–dependent, because Nox2−/− endothelial cells did not show any significant increase of oxidative stress in response to CSE and were also protected against CSE-dependent inhibition of VEGF-induced endothelial cell migration and tube formation (Figure 4A through 4E).

**Nox2 Deficiency Preserves NO Bioavailability in Conditions of Enhanced Oxidative Stress**

NO has been shown to be an important factor involved in ischemia-induced neovascularization. Here we found that nitrite concentration after ischemia is significantly reduced in the plasma of wild-type mice exposed to cigarette smoke. However, Nox2−/− mice are protected against the cigarette smoke–induced decrease of nitrite levels (Figure 5A). Similarly, Nox2−/− mice are also protected against the reduction of plasma antioxidant potential that is induced by cigarette smoke exposure (Figure 5B). In ischemic tissues, eNOS expression was modestly but significantly reduced after cigarette smoke exposure in wild-type but not in Nox2−/− mice (Figure 5C). The importance of NO for the improved neovascularization in Nox2−/− mice was confirmed using the NO inhibitor L-NAME. We found that the beneficial effects of Nox2 deficiency on neovascularization in conditions of increased oxidative stress were lost after treatment with L-NAME in vitro (Figure 5D and 5E) and in vivo (Figure 5F and 5G).

**Discussion**

Our study demonstrates the important role of Nox2-containing NADPH oxidase for the inhibition of ischemia-induced neovascularization in conditions of increased oxidative stress. In contrast, several studies have previously shown that NADPH oxidase–derived ROS can actually promote angiogenesis in different settings. For instance, NADPH oxidase activity is required for hypoxia-stimulated increase in VEGF expression and retinal neovascularization.27 In addition, Nox2-containing NADPH oxidase was shown to play an important role in VEGF-induced angiogenesis52 and in neo-
vascularization after hindlimb ischemia.\textsuperscript{23} These previous studies, however, were performed in young and healthy animals, a situation in which oxidative stress levels would be expected to be relatively low. On the other hand, patients with cardiovascular diseases are typically older and present several risk factors that are associated with increased oxidative stress. In this situation, our results suggest that Nox2-containing NADPH activity is associated with an excessive production of ROS which leads to reduced neovascularization following ischemia.

To mimic a clinical situation of increased oxidative stress, mice in our study were exposed to cigarette smoke before and after the induction of hindlimb ischemia. Cigarette smoking is recognized as the principal modifiable risk factor associated with the development of atherosclerotic diseases.\textsuperscript{24} It has previously been shown to have several negative effects on endothelial function, inflammation, lipid profile, and hemostatic factors.\textsuperscript{24} Reactive oxygen species are known to be involved in smoking-induced endothelial dysfunction.\textsuperscript{28} Moreover, we have previously shown that cigarette smoke exposure is also associated with impaired neovascularization after ischemia,\textsuperscript{14} and that this is at least partly attributable to the generation of ROS.\textsuperscript{18} Here we demonstrate that Nox2 expression is upregulated in ischemic tissues of mice exposed to cigarette smoke, and that this is associated with an important increase of oxidative stress in ischemic tissues after cigarette smoke exposure. These findings are consistent with previous findings indicating that cigarette smoke induces endothelial superoxide anion production via NADPH oxidase activation.\textsuperscript{29} Therefore, our results demonstrate the
critical role of Nox2 for the increase of oxidative stress after cigarette smoke exposure.

We found that lower levels of oxidative stress in Nox2<sup>−/−</sup> mice correlate with improved neovascularization after ischemia, as assessed by blood flow recuperation and capillary density in ischemic tissues. Although this was particularly apparent in mice exposed to cigarette smoke, Nox2 deficiency was consistently associated with improved neovascularization, even in control mice not exposed to cigarette smoke. On the contrary, Tojo et al reported that Nox2<sup>−/−</sup> mice exhibit impaired angiogenesis in response to hindlimb ischemia. These discrepancies might be related to variations in the severity of ischemia that is surgically induced in the mouse hindlimb ischemia model. In fact, the complete and very rapid recovery (within 7 days) of hindlimb blood flow perfusion in Tojo et al suggests much lower levels of ischemia and oxidative stress in ischemic muscles compared to our study. Taken together, our study combined with others suggests a doubled-edge effect of Nox2-derived ROS whereby low levels can serve as signaling agents promoting vascular integrity, whereas higher levels are associated with endothelial dysfunction and impaired neovascularization in response to ischemia.

The mechanisms by which Nox2<sup>−/−</sup> mice are protected against oxidative stress–dependent impairment of neovascularization are potentially diverse. We and others have previously shown that situations of excessive oxidative stress can impair VEGF-induced angiogenesis in endothelial cells. Here we found that mature endothelial cells isolated from Nox2<sup>−/−</sup> mice exhibit lower superoxide levels after CSE exposure. Moreover, reduced superoxide production in Nox2<sup>−/−</sup> endothelial cells exposed to CSE was associated with improved cellular migration and tube formation in response to VEGF. This suggests that Nox2 deficiency can rescue VEGF angiogenic activities in conditions of increased oxidative stress. Recent studies have shown that NO is an essential mediator of endothelial cell migration and VEGF-induced angiogenesis. However, conditions of increased oxidative stress such as cigarette smoking are associated with reduced NO bioactivity. In the present study, we show that plasmatic nitrite levels and eNOS expression in ischemic muscles are significantly reduced after cigarette smoke exposure in wild-type but not in Nox2<sup>−/−</sup> mice. This increased NO bioavailability in Nox2<sup>−/−</sup> mice correlates with an improved antioxidant potential in the plasma. Moreover, the beneficial effects of Nox2 deficiency on neovascularization in vitro and in vivo were lost after treatment with the NO inhibitor L-NAME. Globally, our results suggest that Nox2 deficiency preserves the angiogenic activities of mature endothelial cells in conditions of increased oxidative stress through a reduction of ROS production and a rescue of the VEGF/NO angiogenic pathway.

Recent studies suggest that postnatal neovascularization relies not exclusively on the sprouting of mature endothelial cells in preexisting vessels (angiogenesis), but also involves the contribution of bone marrow–derived circulating EPCs. Although EPCs have been shown to express higher levels of antioxidative enzymes and enhanced protection against oxidative stress compared to mature endothelial cells, recent studies have indicated that EPCs are sensitive to oxidative stress. In the context of cigarette smoking, ROS formation is significantly increased in EPCs whereas the plasma antioxidant level of smokers is reduced and correlates with impaired EPC number and functional activity. In contrast to previous reports in humans, we did not document any significant effect of cigarette smoke exposure on the absolute number of mouse peripheral blood EPCs in the current study. This might be related to inherent differences between species (mice versus humans) or variations in the duration of exposure to cigarette smoke (4 weeks versus lifelong). Nevertheless, our results clearly demonstrate that the functional activities of mouse EPCs (VEGF-induced migration, NO production) are significantly impaired after cigarette smoke exposure. In vitro, this was associated with a significant increase of oxidative stress levels, as demonstrated by DHE staining. Importantly, here we show that Nox2 deficiency can normalize oxidative stress levels, restore NO production, and rescue functional activities of EPCs that are exposed to cigarette smoke.

The present study has several limitations. Cigarette smoke contains more than 4000 known constituents. Although our results demonstrate the important role of oxidative stress for cigarette smoke–induced impairment of neovascularization, it is possible that other mechanisms (eg, cellular toxicity, inflammation) are also involved in that pathophysiology. Another limitation is the measurement of highly reactive compounds, which is always a challenge, especially in vivo. Considering the fact that each assay has its own limitations, here we combined different methods for the assessment of NO and ROS in vivo and in vitro. Finally, there is currently no real consensus on the best markers for the identification of EPCs. Although the combination of hematopoietic stem cell markers (CD34, CD117, CD133) with markers for endothelial cells (Flk-1/KDR, VE-cadherin) is the most recognized and accepted method, whether these cells represent true endothelial precursors or hematopoietic progenitors with angiogenic potential is still a matter of debate.

In summary, our study demonstrates that Nox2-containing NADPH oxidase deficiency protects against ischemia in a clinical situation of increased oxidative stress. The mechanism involves improved neovascularization through a reduction of ROS formation, preserved activation of the VEGF/NO angiogenic pathway, and improved functional activities of EPCs. Our results could contribute to explain the neovascularization deficit that has been associated with different cardiovascular risk factors. Moreover, Nox2-containing NADPH oxidase could constitute a novel therapeutic target to reduce oxidative stress and potentially improve neovascularization in patients with severe ischemic vascular diseases.

**Sources of Funding**

This study was supported by a grant from the Canadian Institute of Health Research (CIHR) to A.R. (R74687). A.R. is a scholar from the Fédération de Recherche en Santé du Québec (FRSQ). P.H. and J.G. are doctoral research awardees from the CIHR. J.T. is a doctoral research awardee from the Fédération de Recherche en Santé du Québec (FRSQ). S.E.M. is a doctoral research awardee from the Heart and Stroke Foundation of Canada.
Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2009;29:1522-1528; originally published online July 2, 2009;
doi: 10.1161/ATVBAHA.109.191437
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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METHODS

Murine ischemic hindlimb model

The protocol was approved by the Comité Institutionnel de Protection des Animaux (CIPA) of the Centre Hospitalier de l’Université de Montréal (CHUM). 6-8 week old Nox2−/− mice on a C57BL/6 background were purchased from Jackson Laboratory (Jackson Mice, Bar Harbor, ME). C57BL/6 mice were used as controls. After two weeks of cigarette smoke exposure, unilateral hindlimb ischemia was surgically induced. The animals were anesthetized with 2% isoflurane, after which an incision was performed in the skin overlying the middle portion of the left hindlimb. After ligation of the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated, and the artery and all side branches were dissected free and excised. The skin was closed with a prolene monofilament (6-0) (Johnson & Johnson, ON, Canada). In some experiments, mice were treated with the NO inhibitor NG-nitro-L-arginine methyl ester (L-NAME 1 mg/ml of drinking water) for the whole duration of the study.

Cigarette smoke exposure

Fourteen days before surgery, mice were started on cigarette exposure (1 cigarette, twice a day) via a smoking machine described previously. All exposures were 10 puffs/cigarette with puff time set at 60 s. Total smoking time for 1 cigarette was 10 min (20 min/day). This exposure is associated with carboxyhemoglobin blood levels that are
similar to those of chronic smokers (8-14%)\textsuperscript{1}. Control mice were restrained in the smoking machine for the same period of time but were not exposed to cigarette smoke.

**Monitoring of hindlimb blood flow**

Hindlimb perfusion was measured with a Laser Doppler Perfusion Imager (LDPI) system (Moor Instruments Ltd., Axminster, UK). After anesthesia with a ketamine-midazolam solution (100 mg/kg-5mg/kg, intraperitoneally), consecutive measurements were obtained after scanning of the same region of interest (leg and foot) with the LDPI. The perfusion signal was split into six different intervals, each displayed in a separate color. Low and/or no perfusion signals were displayed in dark blue, whereas the highest perfusion interval was displayed in red. Color photographs were recorded and analyses were performed by calculating the average perfusion of the ischemic and non-ischemic hindlimb. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the left (ischemic) vs. right (non-ischemic) hindlimb\textsuperscript{1}.

**Immuochemistry**

For immunohistochemistry, whole ischemic hindlimbs were immediately fixed in tissue-fix overnight. After bones had been carefully removed, 3-µm thick tissue transverse sections of the hindlimbs were cut at the level of the gastrocnemius muscle and paraffin-embedded so that the whole leg could be analyzed on each section. Identification of endothelial cells in ischemic muscles was performed by immunostaining for platelet endothelial cells adhesion molecule-1 (PECAM-1 or CD31) with a rat monoclonal
antibody directed against mouse CD31 (Pharmigen, San Diego, CA)\(^1\). Capillaries, identified by positive staining of CD31 and appropriate morphology, were counted by a single observer blinded to the treatment regimen under a 200x magnification to determine the capillary density (mean number of capillaries per square millimeter). Serial sections were cut at three different levels, and representative fields were analyzed by counting the number of capillaries in each field. To evaluate local oxidative stress levels in ischemic muscles, nitrotyrosine immunostaining (Upstate, Lake Placid, NY) and dihydroethidium staining (DHE, Molecular Probes) were used\(^2\). Nitrotyrosine is a reflection of peroxinitrite produced locally in tissues. DHE staining is a semi-quantitative assay to evaluate superoxide formation. The specificity of the reaction was confirmed using muscles of SOD1-deficient mice as positive controls and the cell-permeable superoxide scavenger polyethylene-glycol superoxide dismutase (PEG-SOD, Sigma Aldrich) to insure that the DHE signal is SOD inhabitable (data not shown).

**FACS analysis of peripheral blood Endothelial Progenitor Cells (EPC)**

The percentages of EPC contained in the total viable cell population derived from the peripheral blood was measured by FACS (FACSCalibur flow cytometer, Becton Dickenson, Oakville, Ontario, Canada) using the following fluorescence-coupled endothelial cell markers CD34-FITC and Flk1-PE (eBioscience, CA, USA)\(^3\). Cell phenotypes were determined by the analysis of 300 000 events.
Endothelial Progenitor Cell (EPC) isolation

Mouse bone marrow mononuclear cells were isolated from the femora and tibiae by flushing the bone marrow cavities using culture medium. After red blood cell lysis and washing, bone marrow mononuclear cells were plated on 0.005% fibronectin (Sigma, St Louis, MO) and cultured in medium 200 (Cascade Biologics Portland, OR) supplemented with 18% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and low serum growth supplement (2% FBS, 3ng/ml bFGF, 10 µg/ml heparin, 1µg/ml hydrocortisone, and 10 ng/ml EGF; Cascade Biologics). After 4 days in culture, nonadherent cells were removed by thorough washing with PBS. Adherent cells were stained with 1,1’-dictadecyl-3,3,3’,3’ tetramethyllindocarbocyanine perchlorate-acetylated low-density lipoprotein (DiI-acLDL, 2.5µg/ml for 1 h, Invitrogen) and FITC-labeled lectin BS-1 (Bandeiraeas simplicifolia, 10µg/ml for 1 h, Sigma). Bone marrow EPCs were characterized as adherent cells that were positive for both DiI-acLDL uptake and lectin binding.

Plasma antioxidant potential and nitric oxide (NO) release

Antioxidant concentrations in mice plasma samples were determined using the ‘total antioxidant status assay kit’ (Calbiochem Biochemicals, San Diego, CA, USA). The assay relies on the ability of the antioxidants in the sample to inhibit the oxidation of 2,2’-Azino-di(3-ethylbenzthiazoline sulphonate) (ABTS) to ABTS+ by metmyoglobin, a peroxidase. The amount of ABTS+ is monitored by reading the absorbance at 600 nm. The antioxidants in the sample cause suppression of the absorbance to a degree proportional to their concentration. NO production was determined indirectly by
measuring the concentration of the stable end products nitrate and nitrite using a commercial kit (R&D systems Inc., Minneapolis, MN) based on the Griess reaction⁵.

**Western blot analysis**

For total protein extraction, isolated muscles from whole hindlimbs were rinsed in PBS to remove excess blood, snap-frozen in liquid nitrogen, and stored at -80°C until use. Whole-cell protein extracts were obtained after homogenization of muscles from whole hindlimbs of smoking and non-smoking control and Nox2-deficient mice in ice-cold lysis buffer containing 50mM Tris-HCl (pH 7.4), 0.1 mM ethylene glycol tetraacetic acid, 0.1 mM EDTA, 2µM leupeptin, 1mM phenylmethylsulfonyl fluoride, 1% Nonidet P40, 0.1% sodium dodecyl sulphate and 0.1% deoxycholate (Sigma). 40 µg of protein per sample was separated in reducing 10% polyacrylamide gel and electroblotted on nitrocellulose membranes. The following antibodies were used: 1:500 gp91 (Transduction Laboratories), 1:200 p47 and p67 (Santa Cruz Biotechnology, Santa Cruz), 1:1000 eNOS (Cell signaling), and 1:200 α-tubulin (Santa Cruz Biotechnology, Santa Cruz). Protein expression was quantified by high-resolution optical densitometry (Alpha Imager 2000; Packard Instruments, Perkin Elmer, Boston, MA). Results are expressed as density values normalized to α-tubulin.

**Isolation and culture of aortic endothelial cells**

Endothelial cells from the thoracic aorta were isolated by an explant technique. The thoracic aorta was gently cleaned of periadventitial fat and connective tissue and was opened longitudinally and cut into 2 mm-long rings. The aortic segments of each mouse
were placed on Matrigel (Basement membrane, BD Biosciences) in a 24-well plate and incubated in DMEM supplemented with 10% FBS, 10% Newborn Calf serum, 1% penicillin-streptomycin (Invitrogen), 90 ug/mL heparin (Sigma), 50ug/ml endothelial cell growth supplements (VWR), and 100 U/mL fungizone (Invitrogen) at 37°C in a 95% air/5% CO₂ incubator. The vessel segments were removed once cell outgrowth was observed. Approximately 2-3 weeks later, the cells were detached with dispase (BD Biosciences) and then plated onto 0.1% gelatin– coated 25 cm² flasks. The subsequent passages were performed with 0.25% trypsin-EDTA, and cells were split in a 1:4 ratio.

**Preparation of cigarette smoke extracts (CSE)**

CSE was prepared as previously described⁵. Briefly, two cigarettes (Players Plain no filter, tar: 17mg, nicotine: 1.5mg, carbon monoxide: 12mg) were combusted with a modified syringe-driven apparatus. The smoke was bubbled through 50 ml of DMEM. The resulting suspension was filtered through a 0.20-μm pore filter (Millipore Corporation, Bedford, MA, USA) to remove bacteria and large particles. The filtered CSE was shown to be free of endotoxin (Gel clot LAL, Cambrex). CSE was applied to mouse aortic endothelial cells or EPCs in culture for 16 hours within 30 min of preparation to obtain a final concentration of 10%. This concentration corresponds approximately to exposure associated with smoking 1.5 pack per day⁵. No toxic effect of 10% CSE was found since viability was consistently established to be >80% (trypan blue exclusion). Moreover, using Hoechst-PI staining, we found that exposure to 10% CSE does not increase the rate of apoptosis (2-3%) in endothelial cells (data not shown).
Capillary-like tube formation on Matrigel

Endothelial cells were exposed or not to 10% CSE and 400μM L-NAME. After 16 h, endothelial cells were washed and plated (15 000) in 96-well plates that had been precoated with 60μl of growth factor reduced Matrigel Matrix (Biosciences, San Diego, CA, USA) and cultured at 37°C for 6 h with 50 ng/ml of VEGF. After the different treatments, capillary-like tubes were photographed under a dissecting microscope and all side branches were counted by a single investigator in a blinded manner⁵.

Cell migration assay

Cell migration was assessed using a modified Boyden chamber assay⁵. Polyvinylpyrrolidine-free polycarbonate filter Transwell inserts (6.4mm diameter, 8μm pores; Costar, Cambridge, MA) were coated with 0.1% gelatin. Inserts were placed in a 24-well plate containing medium with 50 ng/ml of VEGF. After 16 h of pretreatment in the presence or absence of 10% CSE and 400μM L-NAME, 15 000 cells were added to the upper chamber of the inserts. Cells were allowed to migrate from the upper to the lower chamber for 6 h at 37 °C. The number of cells that had migrated was counted in six different representative high power (200x) fields per insert (2 inserts/condition). The data are presented as number of migrated cells ± standard error from the mean.

Detection of intracellular superoxide and nitric oxide (NO)

Intracellular generation of O₂⁻ and NO were visualized with the fluoroprobes dihydroethidium (DHE, Molecular probes) and diaminofluorescein-2-diacetate (DAF-2DA, Cell Technology), respectively.
Statistical Analysis

All results are expressed as mean ± SEM. Statistical significance was evaluated by ANOVA. A value of \( p < 0.05 \) was interpreted to denote statistical significance.

REFERENCES


Supplemental Figure I. NADPH oxidase subunit protein expression in ischemic tissues. Representative western blots and quantitative analyses of Nox2, p22, p47 and p67 in ischemic muscles of wild type (WT) and Nox2\(^{-/-}\) mice exposed or not to cigarette smoke (SMK) at day 6 after ischemia. One representative blot of 4 is shown. Data are mean ± SEM. *\(P<0.05\) vs. WT mice, #\(P<0.05\) vs. WT SMK mice.