Sildenafil Increases Endothelial Progenitor Cell Function and Improves Ischemia-Induced Neovascularization in Hypercholesterolemic Apolipoprotein E–Deficient Mice

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

Abstract—Hypercholesterolemia is associated with impaired neovascularization in response to ischemia. Potential mechanisms include defective NO bioactivity and a reduction in the number/function of endothelial progenitor cells (EPCs). Here we tested the hypothesis that sildenafil, a phosphodiesterase 5 inhibitor that increases NO-driven cGMP levels, could stimulate EPC function and improve ischemia-induced neovascularization in hypercholesterolemic conditions. Apolipoprotein E–deficient (ApoE−/−) mice were treated (or not treated) with sildenafil (40 mg/kg per day in water), and hindlimb ischemia was surgically induced by femoral artery removal. Sildenafil treatment led to an improved blood flow recovery, an increased capillary density, and a reduction of oxidative stress levels in ischemic muscles at day 7 after surgery. Sildenafil therapy is associated with an increased activation of angiogenic transduction pathways, including Akt, p44/42 mitogen-activated protein kinase, and p38. In vitro, sildenafil increases cellular migration and tubule formation of mature endothelial cells (human umbilical vascular endothelial cells) in a cGMP-dependent manner. In vivo, ApoE−/− mice treated with sildenafil exhibit a significant increase in the number of bone marrow–derived EPCs. Moreover, the angiogenic activities of EPCs (migration and adhesion) are significantly improved in ApoE−/− mice treated with sildenafil. In summary, this study demonstrates that sildenafil treatment is associated with improved ischemia-induced neovascularization in hypercholesterolemic ApoE−/− mice. The mechanisms involve beneficial effects on angiogenic transduction pathways together with an increase in the number and the functional activity of EPCs. Sildenafil could constitute a novel therapeutic strategy to reduce tissue ischemia in atherosclerotic diseases. (Hypertension. 2009;54:00-00.)

Key Words: sildenafil ■ endothelial ■ progenitor ■ cells ■ angiogenesis hypercholesterolemia

The ability of the organism to develop new blood vessels (neovascularization) constitutes an important adaptive response to vascular occlusive diseases.1 Postnatal neovascularization necessitates the activation, migration, and proliferation of mature endothelial cells (angiogenesis).2 In response to ischemia, hypoxia-inducible factor 1 (HIF-1) and vascular endothelial growth factor (VEGF) have been shown to be critical limiting factors for the induction of angiogenesis.3,4 The importance of NO for endothelial cell migration and VEGF-induced angiogenesis was also demonstrated recently.5,6 However, increasing evidence suggests that postnatal neovascularization relies not exclusively on the sprouting of pre-existing vessels, but also the contribution of bone marrow–derived circulating endothelial progenitor cells (EPCs).7

In young patients and in animal models with young and healthy animals, the neovascularization process is very effective so that blood flow restoration after ischemia is almost complete. Neovascularization, however, is impaired in several clinical situations, which leads to incomplete blood flow restoration and significant residual tissue ischemia. Interestingly, the same risk factors that promote the development of occlusive atherosclerotic diseases are also associated with reduced neovascularization in response to ischemia.8–11 Hypercholesterolemia, an important cardiovascular risk factor, has been shown to be associated with impaired blood flow recuperation and angiogenic response in different animal models.10,12 Moreover, not only native neovascularization but also the response to angiogenic growth factors seem to be reduced by hypercholesterolemia.13 The mechanisms by which hypercholesterolemia impairs ischemia-induced neovascularization are not fully understood. However, it has been proposed that hypercholesterolemia–dependent inhibition of angiogenesis is attributed to a reduction of NO bioactivity.14 Interestingly, NO has been shown to be a critical factor for EPC mobilization,15 and hypercholesterolemia is associated with a reduction in the number and function of EPCs.16,17 Therefore, increasing the activity of NO-dependent pathways in hypercholesterolemic conditions could constitute an attrac-
tive strategy to promote neovascularization and reduce tissue ischemia.

Sildenafil (Viagra) is a cGMP-dependent phosphodiesterase 5 inhibitor. This agent was initially developed for its potent effect on the corpus cavernosum and for treating men with erectile dysfunction. However, the enzyme that sildenafil antagonizes (phosphodiesterase 5) is found in high abundance in most vascular beds, where it is responsible for the breakdown of NO-driven cGMP. Therefore, sildenafil increases cGMP concentrations and is a potent vasodilator.18 For instance, sildenafil can prevent endothelial dysfunction induced by ischemia and reperfusion in humans19 and also has beneficial effects in patients with hypoxic pulmonary hypertension.20

In the present study, we tested the hypothesis that sildenafil can improve ischemia-induced neovascularization and blood flow recuperation in hypercholesterolemic apolipoprotein E (ApOE)–deficient mice. We also investigated potential mechanisms involved in this physiopathology and determined the effects of sildenafil on the angiogenic activities of mature endothelial cells and EPCs.

Materials and Methods
For expanded Material and Methods, please see the online Data Supplement, available at http://hyper.ahajournals.org.

Murine Ischemic Hindlimb Model and the Monitoring of Blood Flow and Blood Pressure
The protocol was approved by the Comité Institutionnel de Protection des Animaux of the Centre Hospitalier de l’Université de Montréal. Six- to 8-week–old ApoE−/− mice on a C57Bl/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). Sildenafil (Pfizer) was dissolved in the drinking water (260 mg/L), and water consumption (average: 4 mL per mouse daily) was monitored to achieve a dose of 40 mg/kg per day. Acute unilateral hindlimb ischemia was surgically induced as described previously, monitored to achieve a dose of 40 mg/kg per day. Acute unilateral hindlimb ischemia was surgically induced as described previously, and hindlimb blood flow was monitored with a laser Doppler perfusion imager system (Moor Instruments).11 Blood pressure was monitored with a BP-2000 tail-cuff pressure instrument (Visitech Systems).

Immunohistochemistry
Identification of endothelial cells was performed by immunostaining for platelet endothelial cells adhesion molecule 1 (or CD31) with a rat monoclonal antibody directed against mouse CD31 (Pharmigen), and capillary density was determined as described previously.11 To evaluate local oxidative stress levels in ischemic muscles, an antibody against nitrotyrosine (Upstate) was used.21

Plasmatic cGMP and C-Reactive Protein Measurements
cGMP concentrations (Cayman Chemical) and C-reactive protein concentrations (ICL) were measured in the plasma of mice at day 7 after surgery using commercial kits.

Western Blot Analyses
Please see the online Data Supplement.

EPC Isolation and Characterization
Seven days after ischemia, mouse bone marrow mononuclear cells were isolated as described previously.22 Bone marrow EPCs were characterized as adherent cells that were positive for both Dil-acLDL (1,1′dioctadecyl-3,3′,3′,3′-tetramethylindocarbocyanine perchlorate acetylated low-density lipoprotein) uptake and lectin binding and

![Figure 1. cGMP concentrations in the plasma of ApoE−/− mice treated with sildenafil (SILD; n=6) and in control untreated ApoE−/− mice (CTL; n=4) at day 7 after surgery. Data are presented as mean±SEM. *P<0.05 vs control mice.](http://hyper.ahajournals.org/

were quantified by the examination of random microscopic fields (×200).

EPC Migration and Adhesion Assays
EPC migration and adhesion were assessed as described previously.22

Human Umbilical Vascular Endothelial Cell Wound Assay and Capillary-Like Tube Formation on Matrigel
Measurement of migration was performed by an adapted wound assay in confluent human umbilical vascular endothelial cells (HUVECs). Capillary-like tube formation on matrigel was assessed as described previously.21

Statistical Analysis
All of the results are mean±SEM. Statistical significance was evaluated by unpaired t-test or ANOVA. A value of P<0.05 was interpreted to denote statistical significance.

Results
Sildenafil Improves Ischemia-Induced Neovascularization in ApoE−/− Mice
Sildenafil therapy was well tolerated by ApoE−/− mice and significantly increased cGMP concentration in the plasma (Figure 1). Systemic blood pressures and cholesterol levels were similar between ApoE−/− mice treated or not with sildenafil (see Table S1, available in the online Data Supplement at http://hyper.ahajournals.org). However, sildenafil therapy was associated with a trend toward a reduction of C-reactive protein concentration in the plasma (P=0.05). Hindlimb perfusion was evaluated postoperatively by serial laser Doppler perfusion imager studies. Similar low levels of blood flow were documented in the different groups immediately after surgery (data not shown). However, as demonstrated on Figure 2A, the Doppler flow ratio was significantly improved at day 7 after surgery in mice treated with sildenafil compared with control mice (55±4% versus 35±4%; P<0.05). At the microvascular level, tissue sections from the ischemic hindlimb muscles were examined histologically to determine capillary density. As shown on Figure 2B, CD31 immunostaining demonstrated a significant increase in capillary density in mice treated with sildenafil compared with control mice (719±39 versus 446±91 capillaries per millimeter squared; P<0.05). Because tissue ischemia is associ-
ated with increased generation of reactive oxygen species, we evaluated the effect of sildenafil treatment on oxidative stress levels using nitrotyrosine immunostaining. As shown in Figure 2C, increased capillary density in ischemic muscles of ApoE−/− mice treated with sildenafil was associated with a significant reduction of local oxidative stress levels.

Sildenafil Increases the Activity of Angiogenic Signaling Pathways and Promotes Angiogenesis in a cGMP-Dependent Manner

We next investigated the effect of sildenafil therapy on the induction of angiogenic signaling pathways in ischemic muscles. As shown in Figure 3, the expressions of upstream signals, such as HIF-1α, VEGF, and phospho-endothelial NO synthase, were similar in ApoE−/− mice treated with sildenafil and in control untreated ApoE−/− mice. However, we found that sildenafil treatment in ApoE−/− mice was associated with a significant induction of downstream angiogenic pathways in ischemic muscles, including phospho-p44/p42, phospho-p38, and phospho-Akt. To better characterize the angiogenic effects of sildenafil, in vitro studies were performed in HUVECs. We found that sildenafil is almost as potent as VEGF to induce cellular migration (Figure 4A and 4C) and promote capillary-like tube formation (Figure 4B and 4D) in HUVECs. Our results suggest that the activity of sildenafil is cGMP dependent, because the increased angiogenic properties of HUVECs are abolished when sildenafil-treated cells are also exposed to the guanylyl cyclase inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one) (Figure 4A through 4D).

Sildenafil Therapy Increases EPC Number and Function in ApoE−/− Mice

To identify additional potential mechanisms involved in the beneficial effect of sildenafil on postnatal neovascularization, we isolated and characterized EPCs from the bone marrow of ApoE−/− mice treated or not treated with sildenafil. EPCs were defined as adherent cells that were positive for both acetylated low-density lipoprotein uptake and lectin binding (Figure 5A). As shown in Figure 5B, the number of EPCs was

![Figure 2. Effect of sildenafil on ischemia-induced neovascularization in ApoE−/− mice. A, Representative results of laser Doppler measurements (left) and quantification of perfusion ratios (right) in ApoE−/− mice treated with sildenafil (SILD; n=15) and in control untreated ApoE−/− mice (CTL; n=9) at day 7 after surgery. A color scale illustrates blood flow variations from minimal (dark blue) to maximal (red) values. Arrows indicate ischemic (left) hindlimbs. B, CD31 immunostaining (left) and quantification of capillary density (right) in ischemic muscles harvested at day 7 in sildenafil-treated and control ApoE−/− mice. Arrows indicate positive staining. C, Nitrotyrosine immunostaining (left) and quantification of relative fluorescence intensities (right) in ischemic muscles of sildenafil-treated and control ApoE−/− mice. Data are presented as mean ± SEM. *P<0.05 vs control mice.](http://hyper.ahajournals.org/)}
significantly increased in mice treated with sildenafil compared with controls (273 ± 18 versus 188 ± 18 cells per field; P < 0.05). We also investigated the effect of sildenafil treatment on the functional activities of EPCs. The migratory capacity (Figure 5C) of EPCs isolated from sildenafil-treated mice was significantly improved compared with controls (28 ± 2 versus 15 ± 1 cells per field; P < 0.05). Moreover, the ability of EPCs to adhere to a HUVEC monolayer activated with tumor necrosis factor-α (Figure 5D) was significantly increased in ApoE−/− mice treated with sildenafil (20 ± 2 versus 10 ± 2 attached cells per field; P < 0.05).

**Discussion**

The present study is the first to document the beneficial effect of sildenafil on ischemia-induced neovascularization in hypercholesterolemic conditions. It has been suggested previously that sildenafil could promote angiogenesis in different pathologies, including ischemic stroke, myocardial ische-
mia-reperfusion injury, and hindlimb ischemia. However, these studies were performed in otherwise young and healthy animals, a situation that does not reflect patients with atherosclerotic diseases who often present several risk factors, including hypercholesterolemia. Therefore, whether sildenafil could successfully promote ischemia-induced neovascularization in these conditions remains to be determined. This could have important implications considering numerous failures of clinical trials that used agents shown previously to be effective in healthy animal models.

Our study also sheds new light on the mechanisms involved in the beneficial effect of sildenafil on neovascularization. We have demonstrated that sildenafil modifies the angiogenic properties of both mature endothelial cells and EPCs. We have shown for the first time that, after ischemia, sildenafil therapy can increase the number and improve the functional activities of bone marrow–derived EPCs.

To investigate the effect of sildenafil in hypercholesterolemic conditions, we used the atherosclerosis-prone ApoE/−/− mouse model. These mice have been shown previously to exhibit impaired neovascularization after hindlimb ischemia. Considering the reduced oral bioavailability and increased elimination of sildenafil in mice compared with humans, the dose that we used here is comparable to that of previous clinical studies for the treatment of pulmonary hypertension in humans. This regimen led to a 3-fold increase in the concentration of cGMP in the plasma of treated mice. We found that sildenafil treatment in ApoE/−/− mice was associated with a significantly improved ischemia-induced neovascularization, as demonstrated by a faster rate of blood flow recuperation after hindlimb ischemia and an increased capillary density in ischemic muscles.

The mechanisms by which sildenafil increases ischemia-induced neovascularization are potentially diverse. We did not document any significant effect of sildenafil on blood pressure or cholesterol levels in the current study. However, our results indicate that sildenafil can act locally in ischemic muscles to activate important angiogenic signals. We found that the expression of angiogenic factors, such as VEGF, HIF-1α, and NO, were not modified in the ischemic tissues of sildenafil-treated mice. However, downstream signal transduction pathways that have been shown to be involved in the angiogenic activities of growth factors, such as VEGF, including phospho-p44/p42, phospho-p38, and phospho-Akt, were significantly increased by sildenafil treatment. Interestingly, it has been shown previously that neovascularization is reduced in conditions of increased oxidative stress and that antioxidant therapies can restore angiogenic properties.

Here we found that sildenafil therapy significantly reduces oxidative stress in ischemic tissues of ApoE/−/− mice. Therefore, it is conceivable that the antioxidant properties of sildenafil could contribute to restore angiogenesis in hypercholesterolemic conditions.

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.7,32,33 However, cardiovascular risk factors (including hypercholesterolemia) are associated with impaired number and functional activity of EPCs.16 Because NO bioactivity has been found to be a critical factor for EPC number and function,15 and considering the fact that hypercholesterolemia-related inhibition of angiogenesis has been linked to a reduction in NO bioactivity,14 increasing the activity of the NO/cGMP pathway could constitute an attractive strategy to rescue EPC function. In this setting, sildenafil could have potential advantages over nitrates, which have been associated with the development of tolerance, cGMP-independent actions, protein nitrosation, and oxidative stress.34 In the present study, we found that the number of EPCs in hypercholesterolemic ApoE−/− mice was significantly increased after treatment with sildenafil. It has been shown previously that the number of EPCs positively correlates with cGMP levels in patients with idiopathic pulmonary hypertension and in control subjects.35 On the other hand, higher serum total cholesterol levels are associated with reduced cGMP concentrations.36 Therefore, our results support the concept that higher cGMP levels after sildenafil treatment in hypercholesterolemic conditions lead to an increase in the number of EPCs.

The present study also clearly demonstrates that sildenafil can positively modulate the functional activities of EPCs. We found that EPCs isolated from sildenafil-treated mice exhibit increased migration capacity and an improved ability to adhere to a HUVEC monolayer. Several mechanisms could contribute to improve EPC functional activities in sildenafil-treated hypercholesterolemic animals. Because oxidized low-density lipoproteins have been shown to impair EPC function by reducing endothelial NO synthase activity,37 increasing cGMP levels might contribute to restore NO-dependent functional activities in EPCs. This would be consistent with a previous study in mature endothelial cells, which showed that a soluble cGMP analogue could rescue integrin expression, cellular migration, and tubule formation in conditions of increased oxidative stress.38 In this regard, modification of oxidative stress levels could constitute another mechanism by which sildenafil influences EPC function. Indeed, the functional activities of EPCs have been shown to be impaired in situations of increased oxidative stress.22,38 Moreover, the number and the functional activity of EPCs directly correlate with serum antioxidant capacity.22 Therefore, the antioxidant potential of sildenafil could contribute to restore EPC number and function in situations of increased oxidative stress, such as hypercholesterolemia.

**Perspectives**

The results of the present study could have important clinical implications. Growth factors and/or progenitor cell supplementation have been proposed as potential angiogenic therapies in patients with ischemic vascular diseases. However, the potential candidates for this type of therapy most often also present several risk factors that are associated with EPC dysfunction and impaired neovascularization. The present study suggests that sildenafil, through its positive effects on angiogenic signals and EPCs, could help restore the neovascularization potential in hypercholesterolemic conditions. Whether similar results might be obtained in other important atherosclerotic conditions, such as aging, diabetes mellitus, and hypertension, remains to be determined. If this is the case, sildenafil could represent a novel therapeutic strategy to reduce ischemia and improve function in patients with different cardiovascular risk factors.

**Sources of Funding**

This study was supported by a grant from the Canadian Institute of Health Research (No. 74687) and a Pfizer Cardiovascular Research Award to A.R. A.R. is a scholar from the Fédération de Recherche en Santé du Québec. P.H. and J.G. are doctoral research awardees from the Canadian Institute of Health Research. S.-E.M. is a doctoral research awardee from the Heart and Stroke Foundation of Canada. J.T. is a doctoral research awardee from the Fédération de Recherche en Santé du Québec.

**Disclosures**

None.

**References**

Sildenafil and ApoE−/− Mice Neovascularization

Dussault et al


Sildenafil Increases Endothelial Progenitor Cell Function and Improves Ischemia-Induced Neovascularization in Hypercholesterolemic Apolipoprotein E–Deficient Mice
Sylvie Dussault, Fritz Maingrette, Catherine Ménard, Sophie-Élise Michaud, Paola Haddad, Jessika Groleau, Julie Turgeon, Gemma Perez and Alain Rivard

Hypertension. published online September 21, 2009;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2009/09/21/HYPERTENSIONAHA.109.139451.citation

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2009/09/18/HYPERTENSIONAHA.109.139451.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

Sildenafil Increases Endothelial Progenitor Cell Function and Improves Ischemia-Induced Neovascularization in Hypercholesterolemic ApoE-Deficient Mice

Sylvie Dussault, Fritz Maingrette, Catherine Ménard, Sophie-Élise Michaud, Paola Haddad, Jessika Groleau, Julie Turgeon, Gemma Perez and Alain Rivard
EXPANDED MATERIAL AND METHODS

Murine ischemic hindlimb model, monitoring of blood flow and blood pressure

The protocol was approved by the Comité Institutionnel de Protection des Animaux (CIPA) of the Centre Hospitalier de l’Université de Montréal (CHUM). Six to eight-week-old ApoE\(^{-/-}\) mice on a C57Bl/6 background were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free isolation facility with a light/dark cycle of 12/12 hours. Sildenafil (Pfizer) was dissolved in the drinking water (260 mg/L) and water consumption (average 4 ml per mouse daily) was monitored to achieve a dose of 40 mg/kg/day. This dose was chosen based on previous animal and human studies, taking into account that sildenafil oral bioavailability is reduced and drug elimination increased in mice compared to man\(^1\). Regular water was used in the control group. Unilateral hindlimb ischemia was surgically induced as previously described and hindlimb blood flow was monitored with a laser doppler perfusion imager (LDPI) system (Moor Instruments Ltd., Axminster, UK)\(^2\). 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. Blood pressure was monitored with a BP-2000 tail-cuff pressure instrument (Visitech Systems, Apex, NC). Each mouse was trained for at least 5 consecutive days (30–60 min of blood pressure measurements) before the beginning of the study. The blood pressure was measured three times per week and averaged.

Tissue preparation and immunohistochemistry

For immunohistochemistry, whole ischemic hindlimbs were immediately fixed in tissue-fix overnight. After bones had been carefully removed, 3 \(\mu\)m 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 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, USA), and capillary density was determined as described previously\(^2\). To evaluate local oxidative stress levels in ischemic muscles, an antibody against nitrotyrosine (Upstate, Lake Placid, NY, USA) was used\(^3\). Intensities of fluorescence were measured and analyzed using computer-based analysis (MetaMorph, Molecular Devices, Downingtown, PA) with the same threshold for all sections under 200X. The specificity of the test was confirmed by preincubating the antibody with 10 mM nitrotyrosine (data not shown).
Plasmatic cGMP and CRP measurements

Cyclic GMP concentrations (Cayman Chemical, Ann Arbor, MI) and CRP concentrations (ICL, Newberg, OR) were measured in the plasma of mice at day 7 after surgery using commercial kits.

Western blot analysis

For in vivo experiments, whole-cell protein extracts were obtained after homogenization of muscles from ischemic hindlimbs of control or sildenafil-treated mice in a lysis buffer containing 62.5 mM ethylenediaminetetraacetic acid; 50 mM Tris-HCL, pH 8.0; 0.4% deoxycholic acid; 1% Nonidet P-40; 0.5 µg/ml leupeptine; 0.5 µg/ml pepstatin; 0.5 µg/ml aprotinin; 0.2 mM phenylmethysulfonfyl fluoride; 0.05 aminothyl benzene sulfonfyl fluoride; and 0.1 sodium vanadate. For in vitro experiments, serum-starved HUVECs were cultured in the presence or absence of 35nM sildenafil and 10µM ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; Sigma) for 30 minutes. After treatments, HUVECs were washed with phosphate buffered saline (PBS) and lysed in the same lysis buffer. Protein concentrations were measured according to the Bradford method and a total of 40 µg of protein per sample was separated in nonreducing 10% polyacrylamide gel and electroblotted on nitrocellulose membranes. The membranes were probed with the following antibodies: Hif-1α (1:500; Novus Biologicals, Littleton, CO), VEGF (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), α-tubulin (1:200; Santa Cruz Biotechnology), Phospho-eNOS (Ser-1177; 1:1000; Cell Signaling Technology, Boston, MA), eNOS (1:500; Santa Cruz Biotechnology), Phospho-Akt (Ser-473; 1:500; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), Phospho-p38 (Thr180/Tyr182; 1:1000; Cell Signaling Technology), p38 (1:1000; Cell Signaling Technology), Phospho-p44/p42 MAPK (Thr202/Tyr204; 1:1000; Cell Signaling Technology) or p42 (1:500, Santa Cruz Biotechnology). Specific proteins were detected by chemiluminescent reaction (GE Healthcare Bio-sciences, Piscataway, NJ) followed by exposure to Hyperfilm ECL (GE Healthcare Bio-sciences).

EPCs isolation and characterization

Seven days after ischemia, 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, 3 ng/ml bFGF, 10 µg/ml heparin, 1 µg/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’ tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (Dil-acLDL, 2.5 µg/ml for 1 h, Invitrogen) and FITC-labeled lectin BS-1 (Bandeiraea simplicifolia, 10 µg/ml for 1 h, Sigma). Bone marrow
EPCs were characterized as adherent cells that were positive for both DiI-acLDL uptake and lectin binding and were quantified by examination of random microscopic fields (200X).

**EPC migration assay**

EPC migration was assessed using a modified Boyden chamber assay. Inserts were placed in a 24-well plate containing medium 200 with 50 ng/ml VEGF. 15 000 cells were added to the upper chamber of the inserts in medium 200. Cells were allowed to migrate from the upper to the lower chamber for 6 h at 37°C. Nonmigratory cells were removed from the upper chamber by wiping the upper surface with an absorbent tip. Cells that had migrated to the lower side of the insert were fixed for 10 min with 3.7% formaldehyde and stained with hematoxylin. After extensive PBS washing to remove excess hematoxylin, the number of cells that had migrated was counted in three different representative high power (200X) fields per insert (2 inserts per mice).

**EPC adhesion to an endothelial monolayer**

A monolayer of human umbilical vein endothelial cells (HUVECs; passage 2–5) was prepared in 24-well plates. HUVECs were pre-treated for 16h with tumor necrosis factor-α (1 ng/ml; BD Biosciences, Mississauga, Canada), fixed and stained with DAPI (0.5 µg/mL; Invitrogen). EPCs were labelled with DiI-AcLDL and 15 000 EPCs were added to each well (2 wells/mice) and incubated for 3 h at 37°C. Non-attached cells were gently removed with PBS and adherent EPCs were fixed with 2% paraformaldehyde and counted in three random fields per well.

**HUVEC Cell culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from Cascade Biologics (Portland, OR, USA) and cultured in medium 200 supplemented with 8% fetal bovine serum (FBS) and low serum growth supplement. Cells were grown at 37°C and 5% CO₂. HUVECs were passaged when they reached 90% confluence and passages 2–5 were used for all experiments.

**HUVEC wound assay**

Measurement of migration was performed by an adapted wound assay in confluent HUVEC. In brief, the cells were grown to near confluence in 24-well plates. Mechanical disruption of the monolayer was realized by scraping with a pipette tip. Cells were exposed to either 0.1% FBS (control), 50ng/ml VEGF, 3.5nM sildenafil or 3.5nM sildenafil and 10µM ODQ for 24 h. The cells were then stained with crystal violet for 15 minutes at room temperature. Migration was assessed using an inverted microscope at a
magnification of 200X by an investigator blinded to the experimental conditions. Three fields per well were evaluated and all experiments were performed in quadruplicate.

**HUVEC capillary-like tube formation on matrigel**

HUVECs were plated in 96-well plates precoated with 50 µl of growth factor reduced Matrigel Matrix (Becton Dickinson Labware, Bedford, MA) and cultured at 37°C for 6h with either 0.1% FBS (control), 50ng/ml VEGF, 3.5nM sildenafil or 3.5nM sildenafil and 10µM ODQ. After the different treatments, capillary-like tubes were photographed under microscope and all side branches in a well were counted by a single investigator in a blinded manner. Each experiment was performed in duplicate for each condition. A branch was defined as a straight cellular segment connecting two cell masses (nodes).

**Statistical analysis**

All results are mean ± sem. Statistical significance was evaluated by unpaired t-test or ANOVA. A value of $P<0.05$ was interpreted to denote statistical significance.

**REFERENCES**

Table S1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ApoE</th>
<th>ApoE + sildenafil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>114±2</td>
<td>113±2*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>79±3</td>
<td>80±2*</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>8.35±0.71</td>
<td>7.40±0.44*</td>
</tr>
<tr>
<td>CRP (ng/mL)</td>
<td>2.70±0.11</td>
<td>2.26±0.06†</td>
</tr>
</tbody>
</table>

* p=ns vs. control ApoE mice
† p=0.05 vs. control ApoE mice