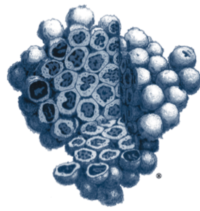

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TRANSLATIONAL AND CLINICAL RESEARCH

Endothelial Progenitor Cell Release into Circulation Is Triggered by Hyperoxia-Induced Increases in Bone Marrow Nitric Oxide

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Key Words. Endothelial progenitor cell • Hyperbaric oxygen • Hyperoxia • Nitric oxide • Neovascularization • Wound healing

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ABSTRACT

Endothelial progenitor cells (EPC) are known to contribute to wound healing, but the physiologic triggers for their mobilization are often insufficient to induce complete wound healing in the presence of severe ischemia. EPC trafficking is known to be regulated by hypoxic gradients and induced by vascular endothelial growth factor-mediated increases in bone marrow nitric oxide (NO). Hyperbaric oxygen (HBO) enhances wound healing, although the mechanisms for its therapeutic effects are incompletely understood. It is known that HBO increases nitric oxide levels in perivascular tissues via stimulation of nitric oxide synthase (NOS). Here we show that HBO increases bone marrow NO in vivo thereby increasing release of EPC into circulation. These effects are inhibited by pretreatment with the NOS inhibitor L-nitroarginine methyl ester (L-NAME). HBO-mediated mobilization of EPC is associated with increased lower limb spontaneous circulatory recovery after femoral ligation and enhanced closure of ischemic wounds, and these effects on limb perfusion and wound healing are also inhibited by L-NAME pretreatment. These data show that EPC mobilization into circulation is triggered by hyperoxia through induction of bone marrow NO with resulting enhancement in ischemic limb perfusion and wound healing. STEM CELLS *2006;24:2309–2318*

INTRODUCTION

Ischemic nonhealing wounds constitute a significant burden on health care systems and are the leading cause of extremity amputation [1]. Surgical solutions fall short in addressing the deficiency in the microvascular blood supply to ischemic extremities. To this end, attention has been focused on angiogenesis, the induction of neovessel growth from locally existing vessels [2]. First described in 1997, the endothelial progenitor cells (EPC) have been the focus of newer research defining the distinct process of vasculogenesis, the growth of neovessels from bone marrow-derived progenitor cells [3]. Once thought to only occur during embryogenesis, vasculogenesis is now an accepted process of neovascularization occurring in the adult [4].

EPC have been shown to home to wounds, tumors, and areas of ischemia and to participate in the establishment of neovasculature [5–8]. During various physiologic stresses requiring healing, EPC trafficking is directed by hypoxic tissue gradients via hypoxic inducible factor 1-α signals inducing stromal derived factor-1 (SDF-1) expression [9], but the physiologic triggers for their mobilization are often insufficient to induce complete wound healing in the presence of severe ischemia [10]. Prior studies demonstrated the requirement of endothelial nitric oxide synthase (eNOS) and its product NO for bone marrow mobilization of EPC, angiogenesis, and wound healing [4, 11, 12]. A number of reports implicate nitric oxide (NO) in both angiogenesis and vasculogenesis [4, 11, 13, 14]. As we elucidate the mechanisms by which EPC are centrally mobilized and home to sites of wounding, discovering methods for exploiting the therapeutic potential of these pathways becomes paramount.

Hyperbaric oxygen therapy (HBO) is a safe, noninvasive modality to enhance wound healing, although the mechanisms for its therapeutic effects are incompletely understood [15–17]. It is known that HBO increases nitric oxide levels in perivascular tissues via stimulation of nitric oxide synthase (NOS) [18, 19]. It is also known that EPC play a central role in wound

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healing and that the EPC response is impaired in ischemic dermal wounds [10]. EPC involvement in wound healing [10], the requirement of NO for peripheral angiogenesis and EPC release from the marrow, and HBO stimulation of NO production [18, 19] in other tissues led us to investigate the possibility that HBO benefits wound healing via NO-mediated mobilization of progenitor cells from the bone marrow.

Materials and Methods

Mice

All procedures were carried out according to the guidelines set forth by The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

For flow cytometric applications, we used transgenic mice expressing the green fluorescent protein (GFP) reporter (Tg(TIE2GFP)287Sato/J; Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) in all cells of endothelial origin (*Tie2*-GFP). In these mice, the *Tie2* promoter, along with an endothelial-specific enhancer located in the first intron of the murine *Tie2* gene, directs GFP reporter gene expression specifically in vascular endothelial cells throughout embryogenesis and adulthood [20]. Naturally occurring *Tie2*, a vascular endothelial-specific receptor tyrosine kinase, binds Angiopoietin-1 (Ang-1). For bone marrow transplantation experiments, we used a similar transgenic murine model expressing the *LacZ* reporter gene (*Tie2*-LacZ mouse) in all cells of endothelial origin (FVB/N-TgN[Tie2LacZ]182Sato; Jackson Laboratory). In these mice, the *Tie2* promoter directs β-galactosidase reporter gene expression. FVB/NJ mice (Jackson Laboratory), the background mouse on which both the *Tie2*-GFP and *Tie2*-LacZ mice were created, were used for all other experiments not requiring reporter gene expression, and as bone marrow recipients in chimeric mice experiments. In all experiments (including direct bone marrow measurements of nitric oxide levels, femoral ligation, wounding, bone marrow transplantation, and laser Doppler imaging), mice were anesthetized with 80 mg/kg ketamine (100 mg/cc Ketaject; Phoenix Scientific, Inc., St. Joseph, MO, http://www.psiqv.com) and 20 mg/kg xylazine (TranquiVed; Vedco Inc., St. Joseph, MO, http://www.vedco.com) given intraperitoneally.

Hyperbaric Oxygen Therapy

To induce tissue-level hyperoxia, mice were subjected to hyperbaric oxygen administration in an animal tabletop chamber (Piersol-Dive, model 4934). The clinically studied Davis Wound Healing Protocol was used, with the animals exposed to 100% oxygen at 2.4 atmospheres absolute (ATA) for 90 minutes [21]. Hyperbaric oxygen treatments were performed after laser Doppler imaging (LDI) flux measurements, so as not to skew LDI flux values due to temporal proximity to the therapy.

In Vivo Bone Marrow Nitric Oxide Measurements

Ten FVB/NJ mice were anesthetized, and an incision was made over the knee joint. The patellar tendon was removed, exposing the patellar surface of the distal femur. An osteotomy was created in the patellar surface of the femur using a 25G beveled needle, allowing access to the marrow space of the femur. Four of the mice received an i.p. injection of N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) 2 hours prior to exposure to HBO treatments. L-NAME is a nonspecific NOS inhibitor, acting on all three isoforms of NOS, including inducible NOS (iNOS), neuronal NOS (nNOS), and eNOS.

NO microsensors were fabricated as previously reported [22] from flint glass micropipettes that were beveled at a ~60° angle after pulling. The micropipettes were partially filled by heating a metal alloy (Wood's metal), leaving a ~100-μm recess at the tip. A 20–40-μm-thick layer of gold then was electroplated into the recess over the metal alloy. A thin layer of Nafion polymer was applied to each electrode by dip coating and then allowed to dry. The NO microsensor was polarized at an oxidation potential of +850 mV relative to an Ag/AgCl reference electrode. Electrochemical oxidation currents were amplified with a sensitive electrometer (Keithly, model 610). The output was low-pass filtered (analog circuit with 5-Hz cutoff) and digitized (two samples per second). The anesthetized animal was placed securely in a hyperbaric chamber, and the electrode was lowered into the marrow space. Continuous NO measurements were taken before, during, and after induction of hyperbaric oxygen therapy for a 10-minute interval.

Multicolor Flow Cytometry Phenotyping

Peripheral blood was obtained via an exsanguinating retro-orbital bleed from anesthetized mice. To isolate the lymphocyte-monocyte fraction, 1 ml of heparin anti-coagulated blood was centrifuged through Histopaque-1083 (Sigma-Aldrich) at 400*g* for 30 minutes. The lymphocyte layer was then removed, washed with phosphate-buffered saline (PBS) twice, and then resuspended in PBS/1% fetal bovine serum (FBS). Cells were then incubated with purified rat anti-mouse CD16/CD32 (FC_{γ}III/II receptor) monoclonal antibody (Mouse BD FcBlock; BD Biosciences, San Diego, http://www.bdbiosciences.com) for 10 minutes at 4°C. Antibody staining of cell surface markers was performed for 1 hour at 4°C using monoclonal antibodies at saturating concentrations. The following monoclonal antibodies were used from BD Pharmingen (San Diego, http://www.bdbiosciences.com/pharmingen): allophycocyanin-conjugated rat anti-mouse CD31, PerCp Cy 5.5-conjugated hamster anti-mouse CD3, and rat anti-mouse CD45. Following antibody incubation, cells were then washed twice with PBS and were resuspended in PBS/1% FBS for analysis on the flow cytometer. DAPI staining was used to screen for viability. One million viable cells were analyzed using an LSR2 multicolor flow cytometer (BD Biosciences). CD3 and CD45 were used to eliminate differentiated immune cells. EPC were defined as cells coexpressing Tie2 (as measured by GFP expression) and CD31/PECAM-1 [9]. Data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR, http://www.treestar.com). Four groups were studied: control mice, HBO-treated mice, mice pretreated with L-NAME followed by HBO, and mice treated by L-NAME alone. Four animals were studied in each group.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Peripheral blood was harvested from anesthetized mice by retro-orbital bleed and collected in heparinized saline. Bone marrow was harvested using the same technique as bone marrow transplantation (detailed below).

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Total RNA was isolated from murine blood (five animals per time point) and bone marrow (three animals per time point) using RNeasy Mini Kit (Qiagen, Valencia, CA, http://www1.qiagen.com) according to the manufacturer's instructions, including an additional DNase digestion to remove any residual DNA. The first strand cDNA was synthesized using the SuperScript III first-strand synthesis system, (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). A 390-bp fragment of vascular endothelial growth factor receptor-2 (VEGFR-2) cDNA was co-amplified with β-actin using the forward primer 5′-AGAACACCAAAAGAGAGAGGAACG-3′ and reverse primer 5′-GCACACAGGCAGAAACCAGTAG-3′ for KDR and the forward primer 5′-ACCACACCTTCTACAATGAGC-3′ and reverse primer GGATGTCAACGTCACACTTGA-3′ for β-actin, and polymerase chain reaction (PCR) was performed at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds and by a final elongation step at 72°C for 2 minutes. Following amplification, the PCR products were then visualized in SYBR Gold (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com)-stained agarose gels. cDNA obtained from mouse livers was used as a positive control for each reverse transcription-polymerase chain reaction (RT-PCR) product. Subsequently, the relative band intensities were analyzed using Kodak 1D Image analysis software (Eastman Kodak Company, Rochester, NY, http://www.kodak.com).

Femoral Ligation Limb Ischemia Model

To induce limb ischemia, the right proximal femoral artery was accessed through an inguinal incision. The femoral nerve was bluntly dissected free of the femoral artery and vein bundle. The artery and vein were ligated proximally and distally with 6-0-coated vicryl suture (Ethicon, Somerville, NJ, http://www.ethicon.com), and a 2–3-mm segment of the vascular bundle between the two ligatures was excised. The skin was then closed with 5-0 nylon (Ethicon) [10]. Limb ischemia was confirmed using laser Doppler perfusion imaging (Moor Instruments, Wilmington, DE, http://www.moor.co.uk). Limb perfusion was assessed daily using laser Doppler perfusion imaging and quantified using Moor Laser Doppler Analysis Software v3.09 (Moor Instruments). The limb was defined as all imaged tissue distal to the inguinal ligament of the mouse. Laser Doppler perfusion imaging was performed in a temperature-controlled facility with weight-based sedation to minimize artifacts due to temperature fluctuations and level of sedation. Four groups were studied: control animals ($n = 7$), HBO animals ($n = 8$), animals pretreated with L-NAME, followed by HBO ($n = 4$), and animals treated with L-NAME alone ($n = 3$).

Assessment of Wound Closure

Bilateral hind limb wounds were induced on the ventral surface of the thigh of the mouse using a 4-mm punch biopsy. A full thickness section of skin was removed, exposing underlying muscle distal to the level of the femoral fold.

Wounds were followed serially with daily digital photographs of the ventral surface of the mice, using an Olympus digital camera. A ruler was included in these photos to allow for calibration of measurements. Images were analyzed using ImageJ software (Imaging Processing and Analysis in Java, NIH). Wound area was measured each day and expressed as a percentage of original wound size.

Bone Marrow Transplantation/Chimera Model

Whole bone marrow was isolated from *Tie2*-LacZ transgenic mice by flushing harvested femurs and tibias with 1% PBS, 2% FBS, and gentamycin. A single cell suspension was created, and the pellet subjected to red blood cell lysis using Red-Cell lysis buffer (Sigma-Aldrich). Cells were washed, counted, and aliquoted for injection.

FVB recipient mice (8–14 weeks of age) were subjected to 9 Gy of total body irradiation via a Shepard Mark IV irradiator, generously provided by Cameron Koch, Ph.D., University of Pennsylvania. Following irradiation, 10×10^6 cells collected from *Tie2*-LacZ bone marrow were reconstituted in 300 μl of PBS and injected into the retro-orbital sinus of the recipient mouse. Mice were allowed to recover for 4 weeks to allow reconstitution.

Following experimentation, PCR for the *LacZ* gene using Jackson Laboratory's genotyping protocol was used to confirm chimerism. At the time of wound harvest, DNA was isolated from bone marrow cells, harvested from femurs, using Qiagen's DNeasy tissue kit. A 315-bp stretch of the *LacZ* gene was amplified using 5′-ATCCTCTGCATGCTCAGGTC-3′ and 3′-CGTGGCCTGATTCATTCC-5′ at 94°C for 3 minutes; 12 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds; followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds; and a final elongation step at 72°C for 2 minutes.

β-Galactosidase Assay for Tissue-Level Detection of Bone Marrow-Derived Endothelial Progenitor Cells

The number of bone marrow-derived (BMD) EPC present in tissue sections was quantified by β-galactosidase assay [10]. Harvested murine tissues were fixed for 10 minutes in 0.5% glutaraldehyde at room temperature and then washed twice with PBS/MgCl solution (10 minutes each wash). The tissues were then incubated with X-gal (Cell Center Facility, University of Pennsylvania) at 37°C overnight, subsequently fixed in Prefer (Anatech Ltd., Battle Creek, MI, http://www.anatechltd.com) for 2 hours at room temperature, and finally embedded in paraffin. Paraffin-embedded serial sections underwent the standard procedures of deparaffinizing and rehydration and were counterstained with nuclear fast red. The number of BMD EPC was quantified by counting β-galactosidase$^+$ cells in serial sections of wound granulation tissue, skeletal muscle underlying the excisional wounds, and distal calf muscle at postoperative day 3 (control mice, $n = 5$; HBO-treated mice, $n = 5$; independently repeated; counts in 10 random high-power fields per section in at least three serial sections).

Statistical Analyses

Statistical analysis of differences was performed using Student's t test. Data were analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA, http://www.microsoft.com). Data are expressed as mean ± SE.

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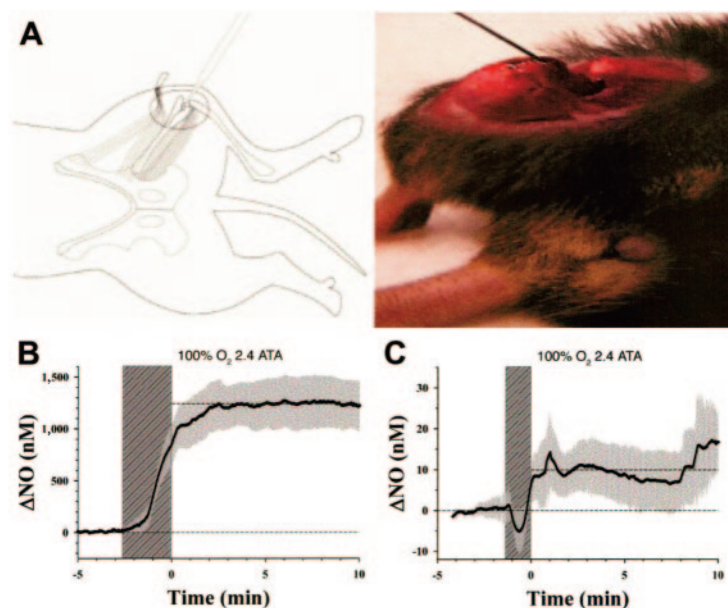

Figure 1. Bone marrow NO production during hyperbaric oxygen (HBO) therapy in mice without femoral ligation or wounding. **(A):** In vivo NO measurement by microsensor inserted into the femur bone marrow in one representative mouse. Left panel, schematic illustration; right panel, photograph. **(B):** NO production measured by NO-specific electrodes inserted into the femur bone marrow of mice undergoing HBO. Mice ($n = 6$) were subjected to 10 minutes of HBO. NO production increases after the onset of pressurization (hatched gray column), reaching a steady state roughly 2 minutes after goal pressure is reached (time = 0 at goal pressure). Solid line represents mean values, with surrounding gray shading representing standard error. **(C):** NO production measured by NO-specific electrodes inserted into the femoral marrow of mice undergoing HBO after pretreatment with L-nitroarginine methyl ester ($n = 4$) 2 hours prior to HBO. HBO-mediated increase in nitric oxide production is inhibited in these animals. Data are analyzed based on the results of three experiments. Abbreviation: ATA, atmospheres absolute; NO, nitric oxide.

RESULTS

Hyperoxia-Induced Stimulation of Bone Marrow NO Production

We studied hyperoxia-induced stimulation of central NO production using Nafion-coated NO microsensors fabricated from flint glass micropipettes in mice without any ischemic stimulus [22]. Microsensors were inserted into the femur marrow space of mice undergoing HBO (Fig. 1A). Mice demonstrated dramatic increases of over 1,000 nM in bone marrow NO production immediately after HBO initiation (Fig. 1B). NO production reached a steady state level approximately 2 minutes after reaching the goal pressure (2.4 ATA) and remained elevated until HBO discontinuation. Increases in NO production were completely inhibited by pretreatment with L-NAME, a nonspecific NOS inhibitor, where no appreciable increase from baseline was noted throughout HBO treatment (Fig. 1C). Normoxic pressure controls that are not expected to significantly change peripheral tissue oxygen levels were performed and did not result in increased bone marrow NO production (data not shown).

Peripheral EPC Populations Following HBO

Along with the increase in bone marrow NO production, tissue-level hyperoxia induced an increase in mobilization of progenitor cells into circulation, assessed by multicolor flow cytometry and molecular analysis of peripheral blood of Tie2-GFP mice expressing the GFP under control of the Tie2 (angiopoietin-1 receptor) promoter. In Tie2-GFP mice, GFP labels cells of the endothelial lineage [23]. HBO treatment approximately doubled the percentage of circulating peripheral cells co-expressing GFP (as a marker of Tie2 expression) and CD31/PECAM-1 (indicative of endothelial-lineage progenitor cells [9]) in mice without any ischemic stimulus. This significant increase in the $Tie2^{+}/CD\ 31^{+}$ cell population was observed as early as 16 hours after a single HBO treatment and was inhibited by pretreatment with L-NAME (Fig. 2A–2H). Exposure to 100% oxygen at ambient pressure, and exposure to a pressure control, 2.8 ATA pressure using a gas containing 7.5% oxygen (so that oxygen partial pressure approximated ambient air), did not stimulate progenitor cell mobilization (data not shown).

Although others have used Tie2 and CD31 to discern EPC in murine models, these markers can also be found on circulating endothelial cells [8, 9]. Therefore, we carried out a second series of trials and gated circulating monocytes for CD31 and also stem cell antigen-1 (Sca1). As was expected, the vast majority of cells expressing both these markers were also GFP-positive, indicating they also expressed Tie2. We found that 97.3 ± 1.2% ($n = 6$) of Sca1/CD31 dual-positive cells from air-breathing control mice exhibited Tie2 activity, and 96.9 ± 1.2% ($n = 6$) of cells obtained 16 hours after mice were exposed to HBO expressed Tie2 activity. All three markers should be found on circulating EPC. The three markers were found on 0.06 ± 0.02% (SEM, $n = 6$) of circulating monocytes in control mice, and in 0.46 ± 0.09% ($n = 6$; $p < .05$) of cells from HBO-exposed mice (data not shown).

We used semiquantitative RT-PCR to evaluate VEGFR-2 (a marker for EPC [9]) mRNA transcripts in blood and bone marrow of mice undergoing daily HBO treatments without any ischemic stimulus. VEGFR-2 mRNA transcript levels in the blood increased nearly threefold by the third treatment (Fig. 2I), and levels in the bone marrow increased nearly fivefold by the fourth treatment (Fig. 2J).

Limb Perfusion and Wound Healing

To evaluate the effects of HBO on limb spontaneous revascularization and ischemic wound healing, we used a murine model of unilateral hind limb ischemia via femoral ligation and subsequent bilateral 4 mm dermal excisional wounding. LDI was used to confirm postoperative ischemia (Fig. 3A, 3B) and monitor quantitative restoration of hind limb blood flow via mean perfusion (expressed as flux). Ischemic limbs in the group treated with HBO showed higher mean flux compared with

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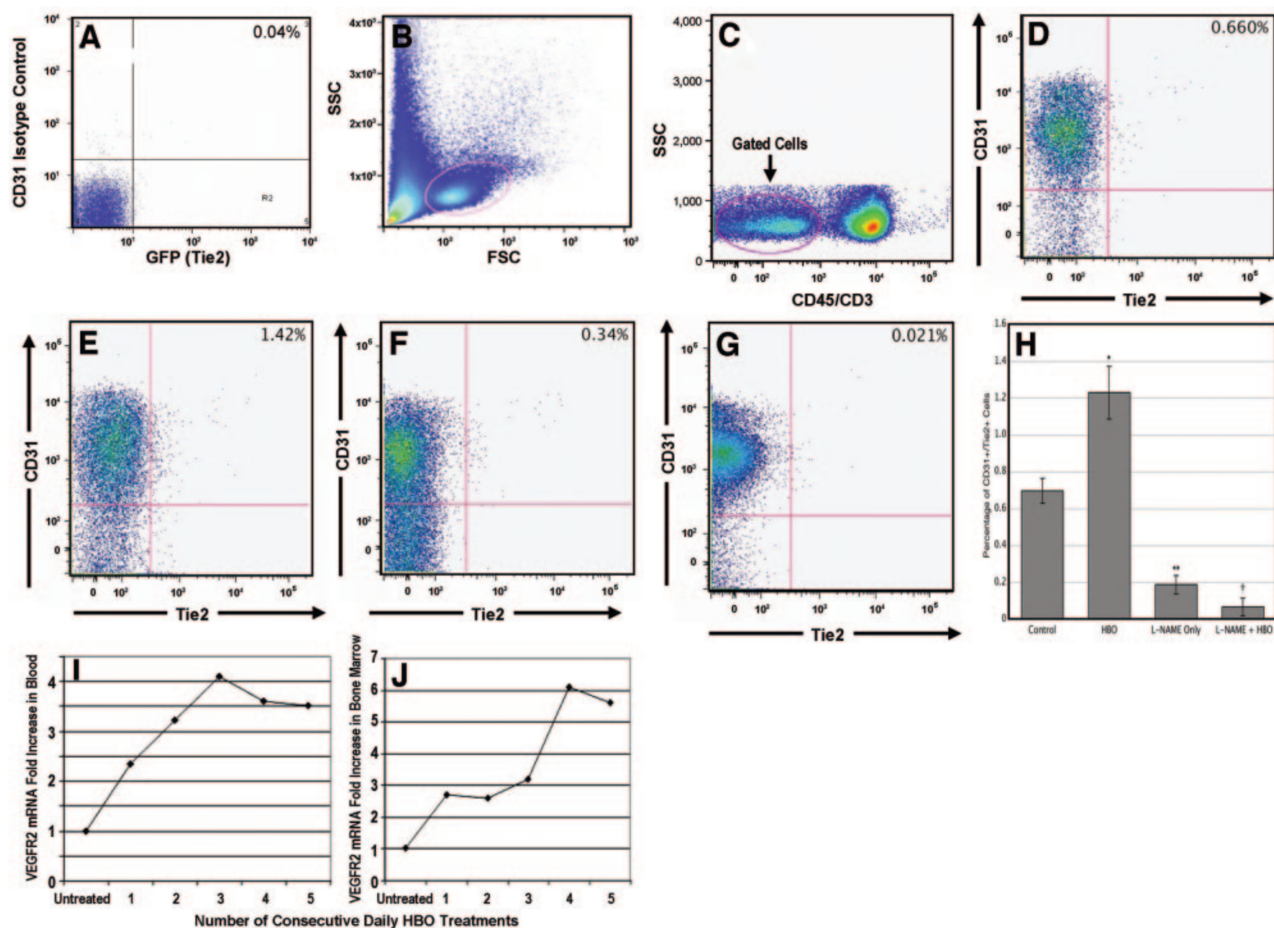

Figure 2. Peripheral mobilization of progenitor cell populations with HBO therapy in mice without femoral ligation or wounding. **(A–H):** Flow cytometry analysis of CD45$^-$/CD3$^-$ cells of the lymphocyte-monocyte population in peripheral blood of FVB/N-TgN (*Tie2*-GFP) Sato transgenic mice. Endothelial progenitor cells defined as *Tie2*$^+$/CD31$^+$; representative populations shown with EPC percentages noted in the *Tie2*$^+$/CD31$^+$ quadrants. **(A):** Isotype control (for CD31). **(B, C):** The gates used to analyze the cells in the CD45$^-$/CD3$^-$ cell population. **(D):** Untreated animals. **(E):** Animals 16 hours after a single HBO treatment. **(F):** Animals treated with L-NAME and harvested 16 hours later. **(G):** Animals treated with L-NAME 2 hours prior to a single HBO treatment, and harvested 16 hours post-HBO treatment. **(H):** Quantitation of *Tie2*$^+$/CD31$^+$ populations (n = 4 for each group). All data are expressed as mean ± SEM based on four experiments. *, HBO-treated mice had a significant rise in the percentage of *Tie2*$^+$/CD31$^+$ cells compared with controls ($p = .04$). **, L-NAME inhibition of NOS resulted in significantly fewer EPC versus both control mice ($p = .003$) and HBO-treated mice ($p = .005$). †, Animals treated with L-NAME followed by HBO treatment also had significantly fewer EPC compared with control mice ($p = .001$) and HBO-treated mice (.004). There was no significant difference between groups treated with L-NAME only and those treated with L-NAME followed by HBO. **(I, J):** reverse transcription-polymerase chain reaction performed for VEGFR2 mRNA transcripts isolated from peripheral blood (n = 5 animals per time point) and bone marrow (n = 3 animals per time point), respectively, for 5 consecutive days. Specific bands were scanned with densitometer. Values expressed as fold increase over baseline (untreated) animals, which are set as 1. Experiments were repeated three times, and similar results were obtained. Data from one representative experiment are shown. Abbreviations: FSC, forward scatter; GFP, green fluorescent protein; HBO, hyperbaric oxygen; L-NAME, L-nitroarginine methyl ester; SSC, sidescatter; VEGFR, vascular endothelial growth factor receptor-2 (VEGFR-2).

untreated ischemic limb group after three consecutive HBO treatments (Fig. 3C, 3D, 3G, 3H, 3K, 3M). Pretreatment with L-NAME inhibited the HBO-mediated improvements in perfusion (Fig. 3E, 3I, 3K, 3M), indicating that HBO enhancement in hind limb flux is NO-mediated. L-NAME alone did not significantly alter the perfusion from untreated levels (Fig. 3F, 3J, 3K, 3M), as expected due to the short half-life of L-NAME, which is long enough to block NO production during HBO (with L-NAME pre-HBO-treatment) but short enough to allow these animals to produce steady-state endogenous NO during the remainder of their postoperative course. Nonischemic limbs showed no difference in perfusion between HBO-treated and untreated animals (Fig. 3L, 3N). The beneficial effects of HBO on limb perfusion and wound healing were thus present only in the setting of pre-existing tissue-level ischemia.

Daily wound area measurements were obtained via digital photography in these HBO-treated mice and untreated controls. Following eight daily HBO treatments, ischemic wounds treated with HBO had significantly smaller wounds (expressed as a percentage of original wound size) than untreated ischemic wounds (Fig. 4A, 4B). The addition of daily L-NAME pretreatment to the HBO-treated animals abolished the healing response enhancement obtained with HBO. The administration of L-NAME alone showed no sig-

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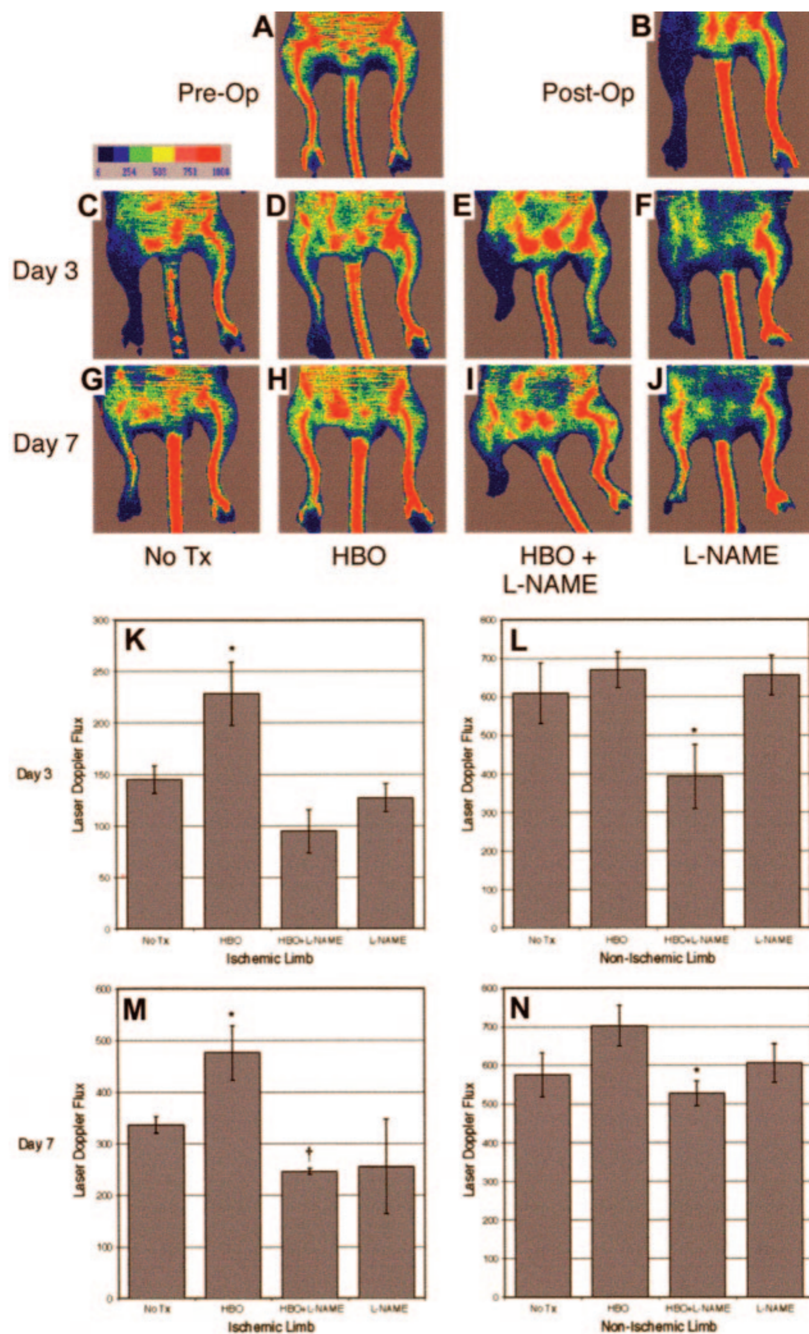

Figure 3. Hind limb perfusion via laser Doppler imaging (LDI) of the ventral surface of hind limbs in mice that underwent unilateral femoral ligation and bilateral hind limb wounding. Color scale with relative laser flux is shown in upper left corner. **(A–J):** Representative images. **(A):** Preoperative LDI, showing good perfusion to both extremities. **(B):** Postoperative LDI, showing severely decreased flow to the hind limb that underwent femoral ligation/excision. **(C):** Untreated animal on postoperative day 3, with persistent ischemia of the hind limb after femoral ligation/excision. **(D):** Animal on postoperative day 3 treated with daily HBO showing return of perfusion to the ligated limb. **(E):** Animal on postoperative day 3 treated with daily HBO, as well as daily L-NAME pre-HBO treatment. Use of L-NAME inhibited the increase in perfusion seen by HBO treatment and the limb developed distal gangrene (tissue loss from ischemia). **(F):** Animal on postoperative day 3 treated with daily L-NAME only. **(G):** Untreated animal on postoperative day 7. **(H):** Animal on postoperative day 7 treated with daily HBO showing increased perfusion of the ischemic hind limb compared with untreated animal. **(I):** Animal on postoperative day 7 treated with daily HBO, as well as daily L-NAME pre-HBO treatment. Use of L-NAME has inhibited the increase in perfusion seen by HBO treatment (ischemic distal limb autoamputation has occurred). **(J):** Animal on postoperative day 7 treated with daily L-NAME only. **(K–N):** Quantitative hind limb perfusion in hind limbs undergoing femoral ligation/excision as measured by laser Doppler flux. All data are expressed as mean ± SEM based on three experiments. **(K):** Postoperative day 3 ischemic hind limb flux. ∗, $p = .03$ versus no treatment, and $p = .005$ versus HBO + L-NAME. **(L):** Postoperative day 3 nonischemic hind limb laser Doppler flux. ∗, $p = .03$ versus HBO-treated limbs, demonstrating L-NAME inhibition of HBO effects. **(M):** Postoperative day 7 ischemic hind limb laser Doppler flux. ∗, $p = .04$ versus no treatment, and $p = .003$ versus HBO + L-NAME. †, $p = .001$ versus no treatment. **(N):** Postoperative day 7 nonischemic hind limb laser Doppler flux. ∗, $p = .02$ versus HBO. Abbreviations: HBO, hyperbaric oxygen; L-NAME, L-nitroarginine methyl ester; Post-Op, postoperative; Pre-Op, preoperative; Tx, treatment.

nificant difference from untreated animals. In all groups (untreated, HBO, HBO + L-NAME, and L-NAME), no significant differences in wound size were noted in the contralateral nonischemic hind limb wounds (Fig. 4A, 4B).

Tissue Level Localization of Progenitor Cells

To investigate whether the observed increases in peripheral EPC populations resulted in a tissue-level increase in EPC, we studied a chimeric mouse model using *Tie2*-LacZ transgenic mice as bone marrow donors to background FVB/NJ mice. This model allowed us to identify cells within peripheral tissue expressing LacZ as BMD EPC [10]. BMD EPC were quantified in serial hind limb sections from chimeric mice undergoing femoral artery ligation and hind limb wounding with or without daily HBO. Representative photomicrographs demonstrating $LacZ^+$ progenitor cells are shown for wound base tissue, proximal (thigh) muscle, and distal (calf) muscle in ischemic limbs with and without HBO (Fig. 5A–5F). Wound granulation tissue showed no difference in EPC numbers after three daily HBO treatments (Fig. 5G). However, the proximal muscle beds demonstrated an unexpected significant decrease in EPC in mice treated with HBO (Fig. 5H). No statistically significant difference in the number of tissue level EPC was seen in distal

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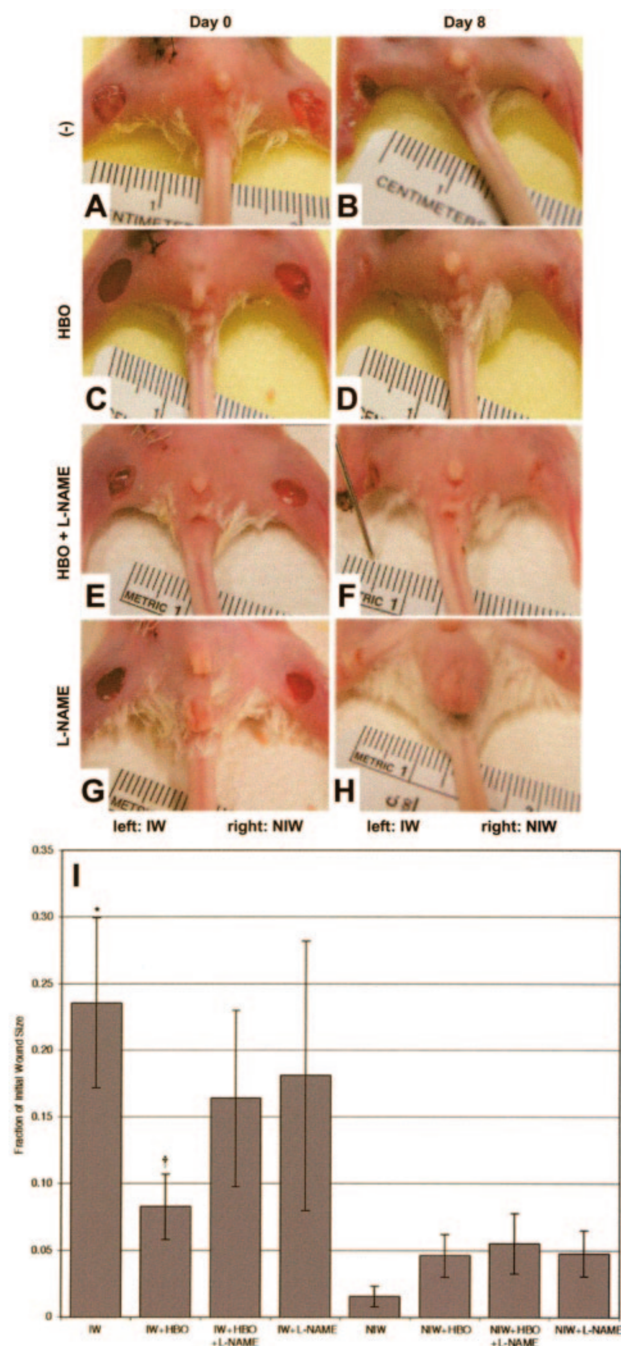

Figure 4. Wound healing rates of ischemic and nonischemic wounds in mice that underwent unilateral femoral ligation and bilateral hind limb wounding. **(A):** Representative photographs of wounds before and after eight daily HBO consecutive treatments. Note that use of L-NAME inhibited the increase in perfusion seen by HBO treatment, and the limb developed distal gangrene (tissue loss from ischemia) **(F)**. **(B):** Fraction of initial wound size measured by digital photography on postoperative day 8 after wounding and after eight daily consecutive HBO treatments according to the Davis Wound Healing Protocol. All data are expressed as mean ± SEM based on three experiments. $*$, $p = .035$ for ischemic wounds treated with HBO versus untreated wounds. No significant differences were seen between the nonischemic wound groups. Abbreviations: HBO, hyperbaric oxygen therapy; IW, ischemic wound; L-NAME, N_{ω}-nitro-L-arginine methyl ester hydrochloride; NIW, nonischemic wound.

ischemic muscles, although a trend toward an increase was seen (Fig. 5I).

Discussion

Our data demonstrate the direct effect of tissue-level hyperoxia on increasing bone marrow nitric oxide production and the circulating pool of endothelial progenitor cells. Previously, NO-mediated EPC release into circulation has been described as the physiologic signaling mechanism to tissue hypoxia, although for unknown reasons, this compensatory response is inadequate in the setting of several ischemic diseases. Using an experimental model that lacks ischemic stimulus, we have determined that hyperoxia induced by clinically safe hyperbaric oxygen exposure paradoxically results in a signaling cascade similar to that induced by tissue hypoxia. By further studying a clinically relevant model of induced limb ischemia and wounding, we have demonstrated that repeated treatments with hyperbaric oxygen increases the rate of wound closure in ischemic wounds and increases return of blood flow to ischemic extremities, also NO-mediated phenomena. These findings provide preclinical validation for the key role of hyperoxia-NO-mediated EPC release in promoting neovascularization and wound healing and for a novel and unexpected method of augmenting compensatory physiologic responses that mobilize progenitor cells into circulation.

Wound closure rate was not significantly changed by hyperoxia in nonischemic limbs, showing lack of toxicity and suggesting that central (hyperoxia-activated bone marrow NOS) and peripheral (ischemic tissue upregulation of SDF-1 for EPC-homing) [9] mechanisms are likely needed in concert to observe the beneficial effects of hyperoxia (HBO)-induced progenitor cell release. Since tissue-level hind limb BMD EPC counts do not appear to significantly increase with HBO, these data suggest that progenitor cell release from the bone marrow may enhance limb revascularization and wound healing by paracrine effects. Alternatively, tissue-level hyperoxia may influence peripheral angiogenesis by distinct and concurrent NO-mediated mechanisms.

Not all reported studies have been consistent as to the NO-mediated effects on BMD EPC. In contrast to our study, NO has been reported to inhibit EPC proliferation [24, 25], and NOS inhibitors have been noted to increase bone marrow stem cell populations, in vitro [26]. These contradictory findings likely indicate that the delicate microenvironment of the marrow is incompletely replicated in vitro. However, our findings are consistent with most prior reports as to the central role of NO in EPC release, angiogenesis, and wound healing.

Murohara et al. showed a significant role for NO in revascularization of ischemic limbs by upregulating NO via L-arginine dietary supplementation [11], whereas Lloyd et al. have demonstrated inhibition of hind limb arteriogenesis by blocking NO production [27]. Yamasaki et al. have shown that topical iNOS gene transfer corrects impaired wound healing in iNOS-deficient mice, demonstrating the importance of local tissue level NO in wound healing [28]. The bone marrow effects of HBO on specific NOS isoforms are unknown and require further study. In addition, the peripheral homing mechanisms that could be exploited to optimize the benefit of systemic progenitor cell release will need to be

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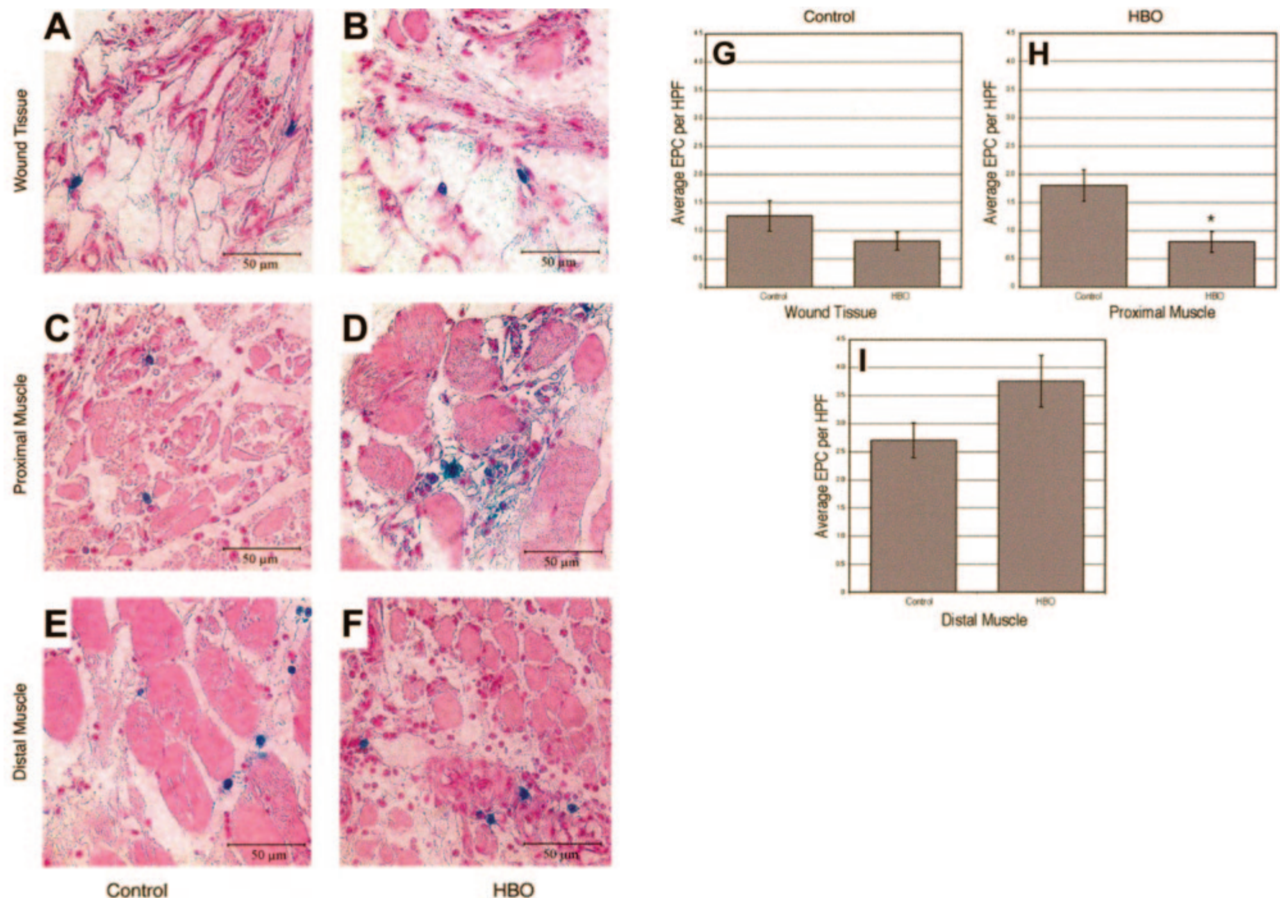

Figure 5. Tissue level localization of EPC in ischemic hind limbs in mice that underwent femoral ligation and hind limb wounding. **(A–F):** Representative ×40 sections from chimeric mice harvested on postoperative day 3 following femoral artery ligation, excision, and wounding. EPC are identified using β-galactosidase staining to localize expression of LacZ reporter containing bone marrow-derived cells. Sections were counterstained with nuclear fast red. **(G–I):** Quantitative measurement of EPC in tissues harvested at postoperative day 3 ($n = 5$ mice for each group, control vs. HBO). **(G):** Ischemic wound base granulation tissue. No difference was seen in the presence of EPC at this tissue level ($p = .09$). **(H):** Proximal thigh muscle underlying the ischemic wound. Animals treated with HBO showed a paradoxical decrease in EPC at this tissue level. *, $p = .003$. **(I):** No significant difference was noted in the presence of EPC in the distal ischemic calf musculature, although there was a trend toward an increase in those animals treated with HBO; $p = .06$. All data are analyzed based on the results of three experiments. Abbreviations: EPC, endothelial progenitor cell; HBO, hyperbaric oxygen; HPF, high-power field.

delineated. Therapeutic SDF-1 upregulation may be a potential target [9]. Hind limb BMD EPC counts decreased in proximal muscle beds. We theorize these findings may be due to a hyperoxia-induced decrease in the proximal peripheral skeletal muscle signaling required for EPC homing (i.e., a reduction in hypoxia-induced SDF-1) [9].

The application of HBO in the setting of NOS inhibition resulted in significantly fewer circulating EPC and significantly decreased ischemic-hind limb perfusion compared with control, and it contributed to distal gangrene of the ischemic leg. As NO is a known scavenger of reactive oxygen species, hyperoxia in the setting of NOS inhibition may create an abundance of reactive oxygen species, which depletes NO, proving detrimental to neovascularization [29]. By serendipity, the combination of HBO, femoral ligation, and NOS inhibition has been identified in this study to yield a consistent murine model of distal hind limb gangrene. In addition, the crucial role of NO in modulating baseline numbers of circulating EPC is further supported by the decrease in EPC observed in the L-NAME-treated mice compared with controls.

We report here for the first time that tissue-level hyperoxia can increase bone marrow nitric oxide and increase the circulating pool of bone marrow-derived endothelial progenitor cells independent of any hypoxic stimulus. However, the beneficial effects of hyperoxia with regard to increased hind limb perfusion and wound healing were unmasked only with the additional stress of surgically induced hind limb ischemia. Why should an additional ischemic stress be necessary? Our data suggest that there are at least two biological processes required for the restoration of perfusion and wound healing in the wounded/femoral-ligated animals, in our mouse model: (a) increased EPC mobilization from bone marrow to the circulation, and (b) homing of circulating EPC to ischemic tissue. Our data suggest that the effect of hyperoxia is mainly to induce an increased mobilization of EPC into circulation by NO-mediated mechanisms at the bone marrow level. Hyperoxia by itself does not regulate the homing of these circulating EPC to the target tissue or organ (herein wound lesion of ischemic hind limb). However, the peripheral tissue hypoxia induces these circulating EPC to home to the low oxygen area (herein the ischemic hind limb).

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The combination of hyperoxia and hypoxia stimuli promote stronger EPC mobilization and, in the presence of the hypoxia signal, homing of the circulating EPC to the ischemic tissue. Such a combination ultimately enhances reperfusion of the ischemic hind limbs (femoral-ligated animals) and promotes wound healing.

BMD EPC mobilization to the periphery by physiologic signals is induced by VEGF-A (from ischemic tissue) that activates bone marrow NOS, producing NO, which activates matrix metalloproteinase-9, enabling release of Kit-ligand and liberating progenitor cells from the marrow [4, 30, 31]. It has been suggested that modulation of this pathway would be beneficial in pathologies such as peripheral arterial disease and coronary artery disease [32, 33]. In this study, the observed hyperoxia-induced effects in circulating BMD EPC were triggered in the absence of any ischemic stimulus. These data are consistent with the known central role of NO in modulating circulating EPC and with our novel finding that hyperoxia drastically increases NO levels within the bone marrow. Our findings demonstrate that tissue-level hyperoxia achieved by nontoxic exposure to hyperbaric oxygen conditions represents an alternative stimulus for the activation of bone marrow NOS, resulting in BMD EPC release into circulation and leading to salutary effects on wound healing and limb spontaneous revascularization.

Until now, hypoxia and some cytokines, such as granulocyte macrophage-colony-stimulating factor (GM-CSF), were the known stimuli to mobilize EPC. Hypoxia triggers incomplete reparative signals, since wound healing is uniformly and consistently worsened by hypoxia despite the mobilization of EPC. GM-CSF is a nonspecific global stimulant of bone marrow associated with many systemic side effects. We herein report the novel finding that hyperoxia induces the liberation of EPC from the bone-marrow pool by an NO-mediated mechanism. In combination with a strong peripheral tissue-level hypoxic stimulus, a functional correlation can be observed with improved ischemic hind limb reperfusion and wound healing. In summary, our study constitutes the new foundation for establishing completely novel clinical paradigms that make use of hyperoxia to specifically induce release of endothelial progenitor cells into circulation from central bone marrow stores. The clinical relevance of these findings are staggering given the number of unsolved clinical pathologies that could benefit from a safe, noninvasive FDA-approved modality for the systemic mobilization of endothelial progenitor cells from central stores. However, before being able to harness the healing potential of these progenitor cells, we will need to use our current knowledge of the homing mechanisms of these cells to direct them to the needed peripheral tissue.

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Disclosures

The authors indicate no potential conflicts of interest.

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