Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1α

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Endothelial progenitor cells (EPCs) are essential in vasculogenesis and wound healing, but their circulating and wound level numbers are decreased in diabetes. This study aimed to determine mechanisms responsible for the diabetic defect in circulating and wound EPCs. Since mobilization of BM EPCs occurs via eNOS activation, we hypothesized that eNOS activation is impaired in diabetes, which results in reduced EPC mobilization. Since hyperoxia activates NOS in other tissues, we investigated whether hyperoxia restores EPC mobilization in diabetic mice through BM NOS activation. Additionally, we studied the hypothesis that impaired EPC homing in diabetes is due to decreased wound level stromal cell–derived factor–1α (SDF-1α), a chemokine that mediates EPC recruitment in ischemia. Diabetic mice showed impaired phosphorylation of BM eNOS, decreased circulating EPCs, and diminished SDF-1α expression in cutaneous wounds. Hyperoxia increased BM NO and circulating EPCs, effects inhibited by the NOS inhibitor N-nitro-l-arginine-methyl ester. Administration of SDF-1α into wounds reversed the EPC homing impairment and, with hyperoxia, synergistically enhanced EPC mobilization, homing, and wound healing. Thus, hyperoxia reversed the diabetic defect in EPC mobilization, and SDF-1α reversed the diabetic defect in EPC homing. The targets identified, which we believe to be novel, can significantly advance the field of diabetic wound healing.

Introduction

Impaired wound healing is a major clinical problem in patients with diabetes and is the leading cause of lower extremity amputation (1). Current therapies have a limited success rate and fall short in addressing the microvascular pathology present in diabetics (2, 3). Poor healing of diabetic wounds is characterized by impaired angiogenesis and vasculogenesis. Vasculogenesis involves the growth of neovessels from BM-derived progenitor cells and contributes to the process of postnatal neovascularization and wound healing (4–6). The BM-derived endothelial progenitor cell (EPC) is a key cell involved in vasculogenesis and home to peripheral tissue in response to ischemia (7, 8). Previous studies have begun to elucidate the mechanisms responsible for the mobilization of EPCs into circulation and their recruitment into areas of peripheral tissue ischemia; however, it remains unknown why the main physiologic stimulus for EPC mobilization and recruitment (i.e., ischemia) fails to induce therapeutic EPC-mediated vasculogenesis and healing in wounds of diabetic hosts.

It has been previously reported that EPCs are mobilized from BM into circulation, home to sites of ischemia, undergo in situ differentiation, and ultimately participate in the formation of new blood vessels (8–10). This EPC mobilization cascade starts with peripheral hypoxia-induced tissue release of VEGF-A and subsequent activation of BM stromal NOS, resulting in increased BM NO levels (11, 12). In this process, eNOS is essential in the BM microenvironment, and increases in BM NO levels result in the mobilization of EPCs from BM niches to circulation, ultimately allowing for their participation in tissue-level vasculogenesis and wound healing (13, 14). At the tissue level, EPC recruitment depends on ischemia-induced upregulation of stromal cell–derived factor–1α (SDF-1α) (15). Impairments in eNOS function have been reported with hyperglycemia, insulin resistance, and in peripheral tissue from diabetic patients (16–20), however, there are no reported prior studies of eNOS function in BM cells in diabetes. In addition, there are no previous studies examining the effects of diabetes on the expression of SDF-1α within cutaneous wounds.

While hypoxia-induced signals for EPC mobilization have been reported, the effect of therapeutic hyperoxia on this pathway is unknown. Induction of hyperoxia, via hyperbaric oxygen therapy (HBO), has been shown to increase NO levels in peripheral tissues via stimulation of NOS (21, 22). Because the generation of NO results in EPC release from the BM and HBO has been shown to stimulate NO production in other tissues, we examined the effect of hyperoxia on activation of NOS in BM. We recently determined that HBO increases BM NO levels via a NOS-mediated mechanism in nondiabetic wild-type mice (23). The effects of diabetes on this hyperoxia-mediated NO increase in the BM has not been previously studied. At a clinical level, current FDA-approved hyperoxia
(i.e., HBO) protocols in diabetic patients have demonstrated inconsistent results in the wound healing response (24–26), underlining the need to elucidate the mechanisms responsible for the therapeutically beneficial effects of HBO.

In this study, we hypothesized that diabetes results in impaired BM eNOS activation and hence reduced mobilization of EPCs from BM into circulation. We then tested whether hyperoxia may specifically enhance BM NOS activity, resulting in increased or restored systemic EPC mobilization in diabetic mice. Specifically, we determined whether a diabetes-associated defect in the production of biologically active eNOS, and thus EPC mobilization, can be reversed by hyperoxia.

We determined that, while hyperoxia increases the mobilization of EPCs into circulation, these cells do not effectively home to diabetic cutaneous wounds. In cutaneous wounds, SDF-1α may function as a homeostatic regulator of tissue remodeling (27). Since cutaneous wounds in diabetic hosts are known to show chronic inflammation and disorganized tissue repair that signifi-
cantly delay healing, we hypothesized that expression of SDF-1α within diabetic peripheral wound tissue is significantly decreased, accounting for the diabetic defect in EPC homing. To examine this question, we studied the expression of the EPC-homing chemokine SDF-1α and the cell source for SDF-1α in diabetic mice. We then tested the hypothesis that exogenous administration of SDF-1α to promote EPC homing to peripheral tissue in order to determine the therapeutic potential for synergistic enhancement of diabetic wound healing.

**Results**

**Impaired phosphorylation of BM eNOS in diabetic mice.** eNOS is essential for EPC mobilization from the BM into circulation (13). In diabetic patients, circulating EPCs are decreased in both number and function, and so we hypothesized that BM eNOS activation is impaired that diabetes-induced changes in mesenchymal stromal cell and lymphocyte populations in the BM might be responsible for the observed downregulation of BM eNOS activation.

**Circulating EPCs are decreased in diabetic mice.** Given the central role of eNOS on EPC mobilization and our results demonstrating impaired eNOS phosphorylation in diabetic BM, we tested the hypothesis that circulating EPCs are decreased in diabetic mice. Our findings demonstrate that diabetic mice have an approximately 50% reduction in circulating EPCs as compared with nondiabetic controls (Figure 1C). Hence, impairment in the phosphorylation of eNOS to its biologically active form likely results in depressed mobilization of EPCs from BM into peripheral circulation.

**Hyperoxia-induced stimulation of BM NO production is attenuated in diabetic mice.** To examine this question, we studied the expression of the EPC-homing chemokine SDF-1α and the cell source for SDF-1α to promote EPC homing to peripheral tissue in order to determine the therapeutic potential for synergistic enhancement of diabetic wound healing.
physiologic stimulus that increases EPC mobilization via a similar NO-mediated mechanism (23). Therefore, we sought to utilize HBO as a tool to augment EPC release into circulation and further study both EPC mobilization and wound-homing mechanisms in the presence of diabetes. We aimed to determine whether hyperoxia stimulates NO production in the BM of diabetic mice via a NOS-mediated pathway and whether this pathway is impaired in diabetic mice. To test this hypothesis, we inserted Nafion polymer-coated NO microsensors into the femur BM space of both STZ-induced diabetic and nondiabetic mice to measure real-time NO levels within the BM prior to and during HBO-induced hyperoxia. As predicted, diabetic mice demonstrated a significantly attenuated rise in BM NO in response to hyperoxia (Figure 1D). Despite this attenuated response, significant increases from baseline were still observed in BM NO levels in response to hyperoxia in the diabetic mice. Specifically, diabetic mice demonstrated an 800-fold increase in BM NO levels during hyperoxic therapy, as compared with a 1,200-fold NO rise in nondiabetic controls. We speculated that this was likely due to a compensatory effect from other NOS isoforms, and therefore we examined the level of both iNOS and nNOS in diabetic mice. As anticipated, the expression of both total iNOS and nNOS protein was upregulated in diabetic mice (Figure 1E). However, we did not observe any changes in the phosphorylation of these NOS isoforms (data not shown). These data suggest that upregulated NOS isoforms require additional stimuli, such as hyperoxia, to be activated in diabetic mice. Consistent with this, hyperoxia-induced increases in NO production were completely inhibited in both the diabetic mice and the nondiabetic controls by pretreatment with N-nitro-l-arginine-methyl ester (l-NAME), a nonspecific NOS inhibitor that inhibits all 3 isoforms of the NOS enzyme (Figure 1F). Hyperbaric normoxic pressurized and hyperoxic nonpressurized control conditions were also studied and demonstrated no increase in BM NO production (data not shown), confirming that hyperoxia is the key stimulus for the BM NO activation.

Increases in BM NO induced by hyperoxia stimulate mobilization of BM EPCs into peripheral circulation in diabetic mice. Physiologically, increased levels of BM NO result in activation of MMP-9 and conversion of kit ligand to its soluble form, ultimately generating EPC release into circulation (11, 31). To study the effects of the hyperoxia-induced rise in NO levels in the BM cavity on mobilization of EPCs into circulation, peripheral blood from diabetic mice was analyzed for EPCs using flow cytometry. We determined whether hyperoxia, induced by HBO, increases the number of circulating EPCs in the peripheral blood of diabetic mice. Specifically, we examined peripheral blood of diabetic mice 18 hours after a single HBO treatment. After excluding dead DAPI-labeled cells, candidate lymphocytes, identified by their typical appearance on forward and side scatter plots, were gated and CD45+/CD3+ cell populations excluded (data not shown). Although EPC markers in humans are well established, the markers that are present on murine EPCs have yet to be precisely defined. Therefore, we used several marker combinations to quantitate circulating EPCs. In independently repeated experiments, EPCs were identified as cells double labeled with either Tie2 and VEGFR2 (32, 33) or (CXC chemokine receptor 4) CXCR4 and VEGFR2 (8, 15, 34). Following treatment with hyperoxia, diabetic mice demonstrate a significant 5-fold increase in circulating CXCR4+/VEGFR2+ EPCs and Tie2-/VEGFR2+ EPCs (Figure 2). Nondiabetic mice treated with HBO showed a similar increase in circulating EPCs, however, the effects of hyperoxia on EPC mobilization were less pronounced as a result of the higher baseline circulating EPC level in the nondiabetic animals. Unlike EPCs, the numbers of circulating lymphocytes were unchanged before and after HBO treatment (Figure 2B), indicating that hyperoxia does not affect lymphocyte mobilization in diabetic mice. In order to specifically determine whether the hyperoxia-induced EPC mobilization is the result of NOS activation, a group of mice were treated with l-NAME prior to HBO treatment. No increase in EPC mobilization following HBO treatment was observed in any of the l-NAME–pretreated animals. In addition, the effect of HBO on EPC mobilization in eNOS−/− mice was examined. No significant changes in EPC mobilization after HBO treatment were observed (Figure 2A), indicating that eNOS is essential.
Synergistic enhancement of EPC mobilization by HBO and SDF-1α in a murine diabetic model. (A) Diabetic mice were divided into 4 groups that received daily wound injections with either SDF-1α or PBS. Half of the mice also received daily HBO. Forty-eight hours after wounding, peripheral blood was analyzed by flow cytometry. Quantification of EPCs was performed for each group. Data are based on 10 experiments. SDF-1α+HBO–treated mice had a significant increase in circulating EPCs compared with other groups (*P < 0.05). SDF-1α- and PBS+HBO–treated groups demonstrated a statistically significant increase as compared with the PBS-treated group (**P < 0.05). (B) Representative dot plots are shown, with number of peripheral blood EPCs noted in each of the CXCR4+/VEGFR2- quadrants. (C) Immunostaining demonstrated a supraphysiologic level of SDF-1α in diabetic wounds after SDF-1α injection compared with nondiabetic wounds. (D) Local administration of SDF-1α results in supraphysiologic systemic peripheral blood SDF-1α level above that present in nondiabetic mice. ELISA demonstrated an increased systemic SDF-1α concentration 2 hours following local wound injection with SDF-1α.

for hyperoxia-induced EPC mobilization. Consistent with this, BM NO production in eNOS−/− mice was significantly reduced (by approximately 40%; data not shown), although not completely obliterated (likely due to the partial compensatory effect of other NOS isoforms, such as iNOS and nNOS), during HBO therapy. Overall, these data demonstrate that hyperoxia, via an eNOS/NOR-mediated mechanism, increases mobilization of EPCs from BM into circulation and reverses the preexisting circulating EPC deficit in diabetes, thus improving the numbers of EPCs potentially available for vasculogenesis and wound healing. However, despite the ability of hyperoxia to increase circulating EPCs, there was no significant increase in the numbers of EPCs homing to diabetic wounds in response to HBO treatment, confirming prior reports that recruitment of EPCs from circulation to peripheral tissue in diabetes is impaired. Thus, we hypothesized that unfavorable local wound conditions, such as decreased levels of the key EPC-homing chemokine, SDF-1α, may explain the diabetic EPC-homing defect and, hence, the disparity between circulating EPC numbers and wound-level EPCs in HBO-treated diabetic animals. SDF-1α expression is decreased in diabetic peripheral cutaneous wounds. Physiologically, SDF-1α is one of the primary chemokines responsible for the mobilization and homing of EPCs to ischemic tissue (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI29710DS1). To identify the type(s) of cell(s) responsible for SDF-1α expression in diabetic wounds, a series of double-staining (SDF-1α and cell type–specific antigen) experiments were performed to examine myofibroblasts (α-SMC actin), epithelial cells (keratin 5), inflammatory cells (CD3/CD4), and ECs (CD31). Epithelial cells and myofibroblasts appeared to be responsible for the downregulation of SDF-1α in diabetic wounds (Figure 3B). Thus, in diabetic wounds, decreased expression of SDF-1α by epithelial cells and myofibroblasts may account for the lack of homing of EPCs to peripheral wounds, despite the increased systemic release of EPCs after HBO treatment. These findings suggest a novel therapeutic target that has not been previously studied in diabetic wound healing.

HBO and SDF-1α synergistically increase circulating EPCs in diabetic mice. Based on our findings that SDF-1α expression in peripheral tissue is decreased in diabetes, we set out to study the effects of exogenous administration of SDF-1α via local wound injections (both alone and in combination with HBO) on EPC mobilization, wound tissue homing, and wound healing in diabetic mice. We hypothesized a potential synergism on EPC tissue-level homing and wound healing using HBO and SDF-1α as combined therapeutically relevant approaches to enhance wound healing in diabetic mice. Our results demonstrated that treatment of diabetic wounds with HBO and SDF-1α together synergistically increased EPC mobilization and homing compared with treatment with HBO or SDF-1α alone, reflecting a potential therapeutic target in diabetic wound healing.
peutic strategies. Interestingly, we observed enhanced EPC mobilization in the SDF-1α + HBO-treated diabetic animals. We had specifically theorized that hyperoxia would enhance EPC mobilization while SDF-1α wound injections would increase homing to diabetic wounds and have a minimal impact on the number of circulating EPCs. Confirming our previous findings, tissue-level hyperoxia induced an increase in the mobilization of EPCs into circulation in wounded diabetic mice, as assessed by flow cytometry of the peripheral blood for cells coexpressing CXCR4 and VEGFR2 (Figure 4, A and B). In these mice, HBO treatment resulted in an approximately 4-fold increase in the percentage of circulating EPCs. Interestingly, local wound injections of SDF-1α resulted in a 2-fold increase in the number of EPCs in circulation. Perhaps most striking, the combination of HBO and peripheral wound SDF-1α administration resulted in a synergistic 11-fold increase in circulating EPCs. The mechanisms responsible for this dramatic synergistic enhancement in EPC mobilization in diabetic mice treated with HBO + SDF-1α require further study. We postulate that alteration of the cytokine milieu of the wound granulation tissue in favor of EPC homing and wound healing may lead to broad paracrine effects from factors released by the wound that, at a systemic level, further enhance BM EPC release. These local factors appear to work in synergism with hyperoxia to greatly increase the systemic mobilization of EPCs. Local wound injections with SDF-1α resulted in supraphysiologic levels of this chemokine within the diabetic wounds, as demonstrated by SDF-1α staining of the wound tissue in diabetic compared with baseline levels of the normal nondiabetic wounds (Figure 4C). In addition, the SDF-1α wound injections resulted in transient supra-physiologic levels of this chemokine within peripheral blood 2 hours (but not 24 hours; data not shown) after local injection (Figure 4D).

SDF-1α enhances EPC homing in diabetic peripheral cutaneous wounds. In order to determine the effect of SDF-1α on homing of the hyperoxia-mobilized EPCs to cutaneous wounds, we carried out BM transplantation experiments, wherein BM from GFP mice was transplanted into γ-irradiated FVB mice. Four groups of wounded diabetic chimeric mice were treated with daily wound injections of PBS, HBO, SDF-1α, or HBO+SDF-1α. After 3 days of treatment, wounds were harvested and analyzed by fluorescent immunostaining of tissue sections with anti-GFP-FITC or anti-VEGFR2-PE Abs. Nuclei were counterstained with Hoescht dye. Recruited EPCs were identified as GFP+/VEGFR2+ cells. (A) Quantification of recruited EPCs in diabetic mice. For each animal, 10 random high-power fields (HPFs, ×100) from 5 serial cross-sections were analyzed, and GFP+/VEGFR2+ cells were quantified relative to the total wound cellularity. Data are based on 3 experiments. SDF-1α+HBO–treated mice had a significant increase in the amount of recruited EPCs compared with other groups (*P < 0.05). SDF-1α–treated animals had a significant increase in amount of tissue EPCs compared with PBS control (**P < 0.05). HBO did not significantly enhance EPC homing to wounds. (B) Representative fluorescent immunostaining of wound sections are shown.
alone, with HBO not significantly changing the number of EPCs (GFP+ /VEGFR2+) present in wound tissue and SDF-1α resulting in a modest but significant 3-fold increase in the number of wound-level EPCs (Figure 5). A synergistic 5-fold increase in the number of EPCs was observed in wound tissue of diabetic mice treated with both HBO and SDF-1α compared with wound tissue of untreated diabetic mice. Similarly, a significantly increased number of CXCR4+ /VEGFR2+ EPCs were observed in wound tissue of diabetic FVB mice (without BM transplantation) treated with both HBO and SDF-1α as compared with wound tissue of untreated control mice (P < 0.05; Supplemental Figure 2). Our data indicate that multi-modality therapy aimed at improving both mobilization and homing of EPCs is an effective strategy to have a significant impact on the number of EPCs available in wound tissue.

The combination of hyperoxia and SDF-1α significantly enhances wound healing in diabetic mice. EPCs play a key role in vasculogenesis and cutaneous tissue repair (6, 40). We hypothesized that increased numbers of EPCs in circulation, along with enhanced EPC homing to wounds, results in improved wound healing in diabetics. We studied diabetic wound closure rates in response to treatment with HBO and SDF-1α alone and in combination. Wounded diabetic mice underwent daily wound injections with SDF-1α, HBO, or HBO+SDF-1α treatments. Only 3 days after initial injury, wound area was found to decrease by 75% in the group treated with SDF-1α + HBO, as compared with a 20% decrease in the PBS controls (Figure 6, A and B). This healing response was greater than the response to either treatment modality alone (P < 0.05), while SDF-1α and HBO treatments alone had significant increases compared with PBS control (**P < 0.05) at day 6. (E) Effect of timing in the initiation of SDF-1α + HBO therapy on wound healing in diabetic mice. Wound closure rates were monitored when treatment was started at days 0, 1, 3, and 5 after wounding and compared with the PBS treated group. Early treatment (days 0 and 1) was necessary to achieve increased closure rate.
Insulin does not increase EPC mobilization or wound healing in diabetic mice. (A) Quantification of circulating EPCs by flow cytometry. Data are based on 3 experiments. (B) Representative dot plots are shown, with number of peripheral blood EPCs noted in each of the VEGFR2/Tie2 quadrants. (C) Minimal effect of insulin on wound healing rate in PBS- or HBO+SDF-1α–treated diabetic mice (n = 10 in each group). Data are based on 2 experiments. No statistical significance was observed in wound closure rates when insulin was introduced to achieve euglycemia.

Discussion

The mechanisms responsible for decreased circulating and wound level EPCs in diabetes have not been previously determined (28–30). This study addressed this fundamental question. Here we show that phosphorylation of eNOS in BM is impaired and that SDF-1α expression by epithelial cells and myofibroblasts in the granulation tissue of cutaneous wounds is decreased in this diabetic murine model. Impaired phosphorylation of eNOS in the BM and decreased wound expression of SDF-1α have a direct impact on EPC mobilization from BM into circulation and EPC homing to wounds, respectively, and can be therapeutically reversed to enhance EPCs available in peripheral wound tissue and improve wound healing. By utilizing HBO to induce tissue-level hyperoxia, we have shown that multiple BM NOS isoforms may be activated, leading to increased NO levels in the BM and hence enhanced mobilization of EPCs into the circulation, thus partially reversing the defect in eNOS activation and EPC release caused by diabetes. In addition, by increasing wound levels of SDF-1α, we have shown that not only is the diabetic BM homing impairment reversed, but also a synergistic increase in EPC mobilization, homing, and wound healing may be achieved when used in conjunction with HBO. The novel targets identified by this study (HBO-mediated EPC release by NOS activation and SDF-1α–mediated EPC homing), along with knowledge about the timing for the initiation of these therapies, carry important clinical relevance for advancement in the field of diabetic wound healing. These data provide the foundation for significant improvements on existing clinical protocols for this significant unsolved medical problem.

Our findings are consistent with prior reports showing that hyperglycemia and diabetes mellitus are associated with impaired eNOS function in a variety of tissues (18, 20, 41). Specifically, hyperglycemia has been found to inhibit eNOS phosphorylation in bovine arterial ECs in vitro by posttranslational modification at the Akt site (18). Furthermore, insulin resistance has been shown to impair eNOS activity by increasing endothelial fatty acid oxidation, a potential mechanism whereby diabetes mellitus results in accelerated atherogenesis and increased cardiovascular disease risk (20). Therefore, our finding that eNOS phosphorylation is impaired in diabetic BM, resulting in depressed EPC mobilization into circulation, builds on recent findings detailing eNOS dysfunction in vitro and in other physiologic systems in diabetic patients (41). In addition, our studies show the inability of insulin treatment to reverse impaired BM eNOS phosphorylation, NO production, EPC mobilization, and wound healing in diabetic mice. This is not surprising, as our results only reflect the short-term effect of
insulin on diabetic wound healing. The effects of long-term treatment with insulin on chronic wound healing in diabetes remain to be studied.

Our data suggest that hyperoxia activates NOS in the BM, thus inducing EPC mobilization into circulation by increasing BM NO production. In diabetic mice, the hyperoxia-induced increase in BM NO was attenuated, likely as a result of impaired eNOS phosphorylation. However, with the induction of hypoxic conditions, other NOS isoenzymes appear to compensate, leading to NO increases in the BM that are substantial and sufficient to reverse the defect in EPC mobilization in our diabetic mouse model.

Although G-CSF and other chemokines have been shown to increase circulating EPCs (42), there is an associated increase in leukocytes resulting in inflammation with the potential for enhanced acute coronary events, which raises questions about the safety and clinical utility of these chemokines (43, 44). This study shows that in the setting of diabetes, hyperoxia increases BM NO levels and stimulates EPC release from the BM into circulation without having a significant impact on the inflammatory cell numbers in circulation. Given that the reduced number of peripheral EPCs is an important contributor to the poor neovascularization and wound healing in diabetic patients, the potential to exploit this mechanism for therapeutic use becomes inherent.

Hyperoxia appears to involve a signal cascade similar to the sequence of events detailing hypoxia-induced EPC release (6–9, 11, 13, 15). The reasons for this seemingly paradoxical response to hyperoxia are unclear, but based on our findings and the reported effects of hypoxia, both hypoxia and hyperoxia result in NOS activation and a subsequent increase in BM NO level. One possible explanation for this response involves the possibility that an oxygen tension sensor, triggered by any perturbation in the oxygen levels, results in NO production and EPC release. Another potential explanation is that both high and low local oxygen concentrations act via distinct mechanisms that ultimately converge on NOS activation. Whatever the reason, it is clear that with hyperoxia, systemic effects of VEGF-A via VEGFR2 mediate NOS activation, whereas with hyperoxia, NO levels rise within minutes and the effects are quickly reversible upon withdrawal of the stimulus, suggesting a direct activation of NOS by a change in oxygen tension. Further studies are needed to more specifically examine this question.

Our data demonstrate that hyperoxia selectively enhances EPC release, resulting in a small but significant improvement in diabetic wound healing but not having a significant impact on wound EPC homing. This may explain the variable clinical effects with hyperoxia, where both high and low local oxygen concentrations act via distinct mechanisms that ultimately converge on NOS activation. Whatever the reason, it is clear that with hyperoxia, systemic effects of VEGF-A via VEGFR2 mediate NOS activation, whereas with hyperoxia, NO levels rise within minutes and the effects are quickly reversible upon withdrawal of the stimulus, suggesting a direct activation of NOS by a change in oxygen tension. Further studies are needed to more specifically examine this question.

Methods

Mice. All procedures were done with approval from the University of Pennsylvania Institutional Animal Care and Use Committee. Six- to twelve-week-old FVB wild-type (FVB/NJ), GFP (FVB/Tg), Tie2-GFP [Tg(TIE2GFp)87 Sato/J] (48, 49), and eNOS−/− (B6.129P2-Nos1tm1iso/J) mice were purchased from The Jackson Laboratory. For all surgical procedures, mice were anesthetized with i.p. injection of 80 mg/kg of ketamine (Phoenix Scientific Inc.) and 20 mg/kg xylazine (Vedco Inc.). For BM transplantation experiments, 1 × 10^7 BM cells from GFP mice were transplanted into γ-irradiated (900 Rad) FVB wild-type mice via tail vein. Reconstitution of transplanted BM in chimeras was achieved at 3 weeks and confirmed by FACScan (>50% BM cells were GFP; data not shown). For local wound SDF-1α injection, SDF-1α protein (R&D Systems) was reconstituted in PBS and injected into the wound base (25 μg/kg).

Induction of diabetes and generation of peripheral wounds. Tie2-GFP and wild-type or chimeric FVB mice at 6–12 weeks of age were treated with STZ (Sigma-Aldrich) to induce diabetes. Mice were rendered diabetic by i.p. administration of 60 mg/kg STZ in 50 mM sodium citrate, pH 4.5, daily for 5 days (50). Control mice were treated with daily injections of citrate buffer. Serum glucose was measured from the mouse tail vein using a glucometer. Once serum glucose reached 250 mg/dl, mice were followed with daily measurements for 1 week prior to use in experiments. Mean serum glucose levels in STZ mice 461 mg/dl with a range of 372–520 mg/dl, while mean control mouse serum glucose levels were 120 mg/dl with a range of 94–135 mg/dl. Wounds were induced on the ventral surface of the mouse thigh using a 4-mm punch biopsy. Full-thickness skin was removed, exposing the underlying muscle.

HBO treatment and in vivo BM NO measurements. For HBO treatment, mice were placed in an animal hyperbaric chamber (Reneau Inc.) and subjected to 100% oxygen at 2.4 atmosphere absolute for 90 minutes (51). To measure continuous real-time BM NO levels, Nafion (Sigma-Aldrich) polymer-coated NO microelectrodes (21, 52, 53) were inserted into the femoral BM cavity as previously described (23). An osteotomy was created on the patellar surface of the murine femur using a 25G bevelled needle, allowing the electrode to be lowered into the BM space when the animal was placed in the hyperbaric chamber. In some mice, pretreatment with t-NAME (40 mg/kg i.p.) (Sigma-Aldrich) was given 2 hours prior to exposure to HBO.

Western blot analysis. Whole BM was isolated from FVB/NJ mice (n = 12/group) by flushing harvested femurs and tibias with PBS/2% FBS. Red blood cells were removed by Red Cell Lysis Buffer (Sigma-Aldrich). Isolated BM cells were then lysed, and protein concentrations were determined by DC protein assay (Bio-Rad). Equal amounts of protein were subjected to 4%–12% SDS gel electrophoresis under reducing conditions. The transferred PVDF membranes were probed with primary Ab (anti-eNOS, anti-phospho-
nOS [Ser1177], anti-iNOS, and anti-nNOS; BD Biosciences) diluted 1:500 in 1% milk in Tris-buffered saline with Tween buffer and then incubated with HRP-conjugated secondary Ab (DakoCytomation). Proteins were visualized using enhanced chemiluminescence (ECL kit; Amersham Biosciences).

**Real-time RT-PCR.** Total RNA was isolated from wound tissues using TRIzol reagents (Invitrogen) in tissue grinders. cDNA was synthesized from 500 ng of total RNA using TaqMan Gold RT-PCR Kit (Applied Biosystems) according to the manufacturer’s protocol. The cDNA samples were diluted 20-fold, and real-time PCR reaction was carried out using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) with 100 μM of primer. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermal cycler conditions were 50°C for 2 minutes and 95°C for 10 minutes to activate or inactivate various enzymes, then 40 cycles each of 15 seconds at 95°C (denaturation) followed by 1 minute at 59°C (annealing and extension). The β-actin plasmid was used as standard DNA. All standards and samples were assayed in triplicate. The threshold cycle values were used to plot a standard curve. All samples were normalized to the relative levels of β-actin (which was set as “1”), and results were expressed as fold-increase. Primers were designed using Primer Express software (version 2.0; Applied Biosystems) as follows: SDF-1α, 5′-CCAGAGCCAGTCAAGCAT-3′ and 5′-TGGTGAAGATTCTGAGTGA-3′; β-actin, 5′-ACGGCCAGTGCTACATCTTG-3′ and 5′-CAAGAAGGAGGGCTGGAAGA-3′. ELISA. Mouse serum SDF-1α concentration was measured by Quantikine mouse SDF-1α ELISA kit (R&D Systems) based on the manufacturer’s protocol.

**Flow cytometry.** Mobilization of EPCs into circulation was studied using flow cytometry as previously described (23). Cells isolated from mice were incubated with various Abs (BD Biosciences). Isotype-matched mouse immunoglobulins served as controls. One million viable cells were scanned, and lymphocytes were gated and analyzed using an LSR II multicolor flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (version 6.4.3; TreeStar Inc.).

**Histocchemistry.** For immunostaining, paraffin-embedded serial sections (5 μm) first underwent standard deparaffinization and rehydration procedures and were then probed with various Abs. To assess wound-SDF-1α cells, sections were double stained with FITC-conjugated anti-SDF-1α and PE-conjugated tissue-specific Abs (eBiosciences). To detect the blood vessels in wounds, sections were stained with FITC-conjugated anti-VEGFR2 Ab. For examination of wound EPC recruitment in the BM transplantation experiment, sections were double stained with FITC-GFP and PE-VEGFR2 (BD Biosciences). Nuclei were counterstained with Hoescht dye. Masson's trichrome staining and H&E staining were performed using standard methods, and all reagents were from Sigma-Aldrich. Tissue sections were analyzed using ImageJ software (version 6.0; NIH) to quantitate fluorescent intensity. In trichrome-stained slides, blue stain (collagen content) was also quantitatively analyzed using ImageJ.

**Assessment of peripheral wound healing.** Initial wound surface area was recorded, and the healing of wounds was recorded daily with digital photographs with daily digital photographs using an Olympus digital camera. Photographs contained an internal scale to allow for standard calibration of measurements. Wound area was quantified using ImageJ software and was expressed as the percentage of original wound size. Wounds were observed for 6 days, and mice were sacrificed at the conclusion of the experiment.

**Statistics.** All data is expressed as mean ± SEM. Statistical analysis was carried out using paired 2-tailed Student’s t test and ANOVA procedures. P < 0.05 was considered statistically significant.

**Acknowledgments**

This work was supported by NIH grants 1-R01-DK071084 and K01-HL073145 and by University of Pennsylvania Institutional Research Funds.

Received for publication July 14, 2006, and accepted in revised form February 6, 2007.

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