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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.

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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 new vessels 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 homing in cutaneous wounds (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 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 in response to ischemia; however, it remains unknown why the main physiologic stimulus for EPC mobilization and recruitment (i.e., ischemia) fails to induce therapeutic EPC-mediated neovascularization 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 therapeutic 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-
Patients, circulating EPCs are decreased in both number and function of diabetic wound healing. To examine this within diabetic peripheral wound tissue is significantly decreased, accounting for the diabetic defect in EPC homing. To examine this compensation response is inadequate in the setting of diabetes-induced changes in mesenchymal stromal cell and lymphocyte populations in the BM might be responsible for the decreased eNOS activation in diabetic mice (28–30). To examine this question, BM was isolated from streptozocin-induced (STZ-induced) diabetic and nondiabetic mice and analyzed via Western blot for levels of total and phosphorylated eNOS protein. Although no changes in the amount of total eNOS protein were observed, the levels of biologically active phosphorylated eNOS protein were decreased in diabetic mice as compared with nondiabetic controls (Figure 1A). To investigate whether the decreased eNOS activity might reflect a change in cellular composition within BM of diabetic mice, we studied the constitution of EPCs, stromal cells, HSCs, and lymphocytes in the BM of STZ-induced diabetic versus nondiabetic mice. The EPC and HSC populations remained constant while the mesenchymal stromal cell and lymphocyte populations demonstrated a slight decrease (by approximately 27% and 26%, respectively) in diabetic compared with nondiabetic mice (Figure 1B). Thus, it is possible 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 approximate 50% reduction in circulating EPCs as compared with nondiabetic mice (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. Physiologically, the NO-mediated EPC release into peripheral blood occurs in response to tissue-level hypoxia (6), although this compensatory response is inadequate in the setting of diabetes and results in severe defects in neovascularization and wound healing. Interestingly, we have determined that hyperoxia is a non-

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| NO-dependent EPC, not lymphocyte, mobilization is enhanced by hyperoxia. Flow cytometry quantification of circulating peripheral blood EPCs (CXCR4+/VEGFR2+) (A) and lymphocytes (B) in diabetic FVB and eNOS-/- mice and peripheral blood EPCs (Tie2+/VEGFR2+) in Tie2-GFP (H) mice. Data are based on 6 (A and B) and 12 (H) experiments. Mice were treated with or without HBO (HBO+ and HBO-, respectively) or with L-NAME + HBO. **P < 0.05; ***P < 0.005. (C–G and I–P) HBO significantly increased circulating EPCs, while L-NAME inhibited this effect. Representative dot plots with number of circulating EPCs in peripheral blood of diabetic FVB (C–G) and nondiabetic (I–L) and diabetic (M–P) Tie2-GFP mice are shown. (C, I, and M) HBO+; (D, J, and N) HBO–; (E, K, and O) L-NAME, and (F, L, and P) L-NAME+HBO. (G) Isotype control (VEGFR2/CXCR4).

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...
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 diabetes. 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 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 12 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 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...
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/NO-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 (8, 15, 35). SDF-1α expression is known to be induced in a wide variety of cell types in response to stimuli such as stress and injury (36–38). The role of SDF-1α in diabetes-related chronic wounds has not been previously studied. Recent studies have indicated that in diabetes, the impaired neovascularization and delayed healing of cutaneous wounds is at least in part due to the reduced number and function of EPCs (28, 30, 39), but it is likely that other tissue-level factors contribute to poor wound healing in diabetes. To examine this issue, we investigated whether the diabetic phenotype is associated with decreased expression of SDF-1α in wound granulation tissue. Peripheral wound tissue from both diabetic and nondiabetic mice at various time points (days 5, 7, and 9 following STZ treatments) was harvested and examined using quantitative real-time RT-PCR. Our results demonstrate that SDF-1α decreased significantly (~50%) at day 9 following STZ treatments (Figure 3A). Moreover, fluorescent microscopy for cells expressing SDF-1 was performed on wounds 10 days after STZ treatment and 24 hours after creation of initial wound. Approximately half as many cells from diabetic wounds expressed SDF-1α as compared to wound cells from nondiabetic mice.
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 Hoeschs 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, SDF-1α + HBO, or SDF-1α + HBO 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 < 0.001 at days 1–5; **P < 0.05 at day 6). Diabetic mice treated with either HBO or SDF-1α demonstrated significantly improved wound healing over PBS controls at days 2, 3, and 5 (**P < 0.05). (B) Representative wounds at days 0, 3, and 6 are shown for each group. (C) Trichrome staining of wound tissues at day 6. Collagen was stained as blue. (D) Quantification of collagen content. Data are based on 5 scanned slides in each group at day 6. Data are based on 3 experiments. SDF-1α + HBO–treated mice demonstrated a significant rise in wound vessel density and collagen deposit compared with individual treatments 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.

mice, we examined wound closure rates when HBO + SDF-1α treatment was initiated on days 0, 1, 3, and 5 following wounding. Our results show that in order to influence wound healing, early application of HBO + SDF-1α is necessary, as initiation of treatment either at initial wounding or 1 day after wounding is effective, while delayed treatment (day 3 or 5) results in the loss of accelerated wound closure rates (Figure 6E).

Insulin is insufficient to restore impaired diabetic wound healing. As insulin is a standard therapy for type I diabetes, we examined the effects of insulin on impaired eNOS/NO/EPC mobilization/wound healing in diabetic mice. STZ-induced diabetic mice were treated with insulin (6 U/kg NPH [isophane] insulin i.p. twice per day) for the duration of each experiment. Interestingly, insulin alone had little effect in reversing impaired BM eNOS phosphorylation (Figure 1A), BM NO production (Figure 1D), or EPC mobilization (Figure 7, A and B) in diabetic mice. Similarly, insulin treatment failed to improve impaired wound SDF-1α production (data not shown) and wound healing rates (Figure 7C). When combined with HBO + SDF-1α therapy, insulin treatment did not further accelerate the wound healing rate. Our data indicate that short-term insulin treatment has minimal effects on improving diabetic wound healing.

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 EPC 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 therapy.
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 hyperoxic 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 hypoxia, 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 on wound healing reported with HBO treatment alone. We postulate that the HBO-mediated enhancement of wound healing is multifactorial. Likely there are systemic paracrine effects due to increased circulating EPCs that in some circumstances may be sufficient to modify the wound healing cascade as previously described (23). An osteotomy was created on the patellar surface of the murine femur using a 25G beveled needle, allowing the electrode to be lowered into the BM cavity as previously described (23). An osteotomy was created on the patellar surface of the murine femur using a 25G beveled needle, allowing the electrode to be lowered into the BM cavity as previously described (23). An osteotomy was created on the patellar surface of the murine femur using a 25G beveled needle, allowing the electrode to be lowered into the BM cavity as previously described (23).

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-

**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<sup>+/−</sup> (B6.129P2-Nos<sup>3<sub>tm1Unc</sub></sup>/J) mice were purchased from The Jackson Laboratory. For all surgical procedures, mice were anesthetized with an 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<sup>7</sup> 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 beveled 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 l-NAME (40 mg/kg i.p.) (Sigma-Aldrich) was given 2 hours prior to exposure to HBO.

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**Conclusion**

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 1% milk in Tris-buffered saline with Tween buffer and then incubated with various Abs (BD Biosciences). Isotype-matched mouse flow cytometry as previously described (23). Cells isolated from mice were results were expressed as fold-increase. Primers were designed using Primer 3. Phillips, T.J. 1994. Chronic cutaneous ulcers: etiology


