Hyperbaric oxygen therapy activates hypoxia-inducible factor 1 (HIF-1), which contributes to improved wound healing in diabetic mice: HBO therapy activates HIF

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Hyperbaric oxygen therapy activates hypoxia-inducible factor 1 (HIF-1), which contributes to improved wound healing in diabetic mice

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ABSTRACT

Hyperbaric oxygen (HBO) therapy has been used as an adjunctive therapy for diabetic foot ulcers, although its mechanism of action is not completely understood. Recently, it has been shown that HBO mobilizes the endothelial progenitor cells (EPCs) from bone marrow that eventually will aggregate in the wound. However, the gathering of the EPCs in diabetic wounds is impaired because of the decreased levels of local stromal-derived factor-1α (SDF-1α). Therefore, we investigated the influence of HBO on hypoxia-inducible factor 1 (HIF-1), which is a central regulator of SDF-1α and is down-regulated in diabetic wounds. The effects of HBO on HIF-1α function were studied in human dermal fibroblasts, SKRC7 cells, and HIF-1α knockout and wild-type mouse embryonic fibroblasts using appropriate techniques (Western blot, quantitative polymerase chain reaction, and luciferase hypoxia-responsive element reporter assay). Cellular proliferation was assessed using H^3-thymidine incorporation assay. The effect of HIF in combination with HBOT was tested by inoculating stable HIF-1α-expressing adenovirus (Adv-HIF) into experimental wounds in db/db mice exposed to HBO. HBO activates HIF-1α at several levels by increasing both HIF-1α stability (by a non-canonical mechanism) and activity (as shown both by induction of relevant target genes and by a specific reporter assay). HIF-1α induction has important biological relevance because the induction of fibroblast proliferation in HBO disappears when HIF-1α is knocked down. Moreover, the local transfer of stable HIF-1α-expressing adenovirus (Adv-HIF) into experimental wounds in diabetic (db/db mice) animals has an additive effect on HBO-mediated improvements in wound healing. In conclusion, HBO stabilizes and activates HIF-1, which contributes to increased cellular proliferation. In diabetic animals, the local transfer of active HIF further improves the effects of HBO on wound healing.

Diabetic foot ulcers represent a chronic complication of diabetes with heavy consequences for both patients and society.[1] Beside the standard available therapeutic options such as offloading, debridement, antibiotics, and improvement of circulation, several therapeutic strategies have been investigated and approved, including living skin equivalents and growth factors. However, even with the best clinical care, up to 10% of patients with diabetic foot ulcers have to undergo amputation.[2,3] Therefore, additional therapies for these patients are greatly needed. Hyperbaric oxygen (HBO) therapy has been used to treat difficult cases for decades,[4] although its basis is still not completely understood. HBO activates several mechanisms that contribute to wound healing,[5] including increased fibroblast proliferation[6] and collagen synthesis,[7] growth factor production,[8–10] improved anti-bactericidal capacity,[11] and the stimulation of angiogenesis.[12] Recently, it has been shown that HBO activates the release of endothelial progenitor cells (EPCs) from the bone marrow that eventually congregate in the wounds and contribute to angiogenesis and wound healing.[13] However, in diabetic animals, EPC homing is impaired at the wound level because of a defect in stromal-derived factor-1α (SDF-1α), which is a cytokine released in the characteristically hypoxic wound environment.[14] Additional inoculation of SDF-1 in diabetic wounds has been shown to dramatically improve healing during HBO.[15]

Adaptive responses of cells to hypoxia are mediated by hypoxia-inducible factor 1 (HIF-1), which is a heterodimeric transcription factor composed of two subunits: HIF-1 alpha and HIF-1 beta. Regulation of HIF-1 activity is critically dependent on the hydroxylation of the alpha subunit in normoxia by prolyl hydroxylases (PHDs), which allows the binding of von Hippel–Lindau tumor suppressor protein (pVHL), and further leads to ubiquitylation and degradation of the alpha subunit.[16] Under hypoxic conditions, HIF-1 alpha is stabilized against degradation, dimerizes with HIF-1 beta, and binds to hypoxia-responsive elements (HREs) in the

promoters of several genes (approximately 100), including SDF-1, which enables the cells to adapt to reduced oxygen availability.[17,18] HIF-1 plays a pivotal role in wound healing[19] and is down-regulated in diabetic wounds.[20]

Therefore, we investigated the potential effect of HBO on HIF-1 function. Moreover, we studied the effect of HIF-1 correction on the outcome of HBO in experimental diabetic wounds.

MATERIALS AND METHODS

Cell cultures and reagents

Human dermal fibroblasts (HDFs) (PromoCell, Heidelberg, Germany) and mouse embryonic fibroblasts (HIF+/+ and HIF–/– MEFs) (kindly provided by Prof. L. Poellinger, Karolinska Institute, Sweden) were cultured in Dulbecco's Modified Eagle's medium (DMEM) medium containing fetal bovine serum (FBS) plus antibiotics (penicillin and streptomycin) (Life Technologies, Stockholm, Sweden). The human SKRC-7 cells, originating from a renal carcinoma from a patient
with functionally inactive VHL, was kindly provided by E. Oosterwijk (Nijmegen, The Netherlands) and maintained as described.[21] All of the cells were maintained in a humidified atmosphere with 5% oxygen at 37 °C.

HBO treatment

For the in vitro experiments, the cells, grown in 60-mm plates without lid, were exposed to HBO (100% oxygen at 2.5 atmospheres absolute [ATA]) for 1 hour in an HBO chamber, while the cells used for the control were simultaneously placed outside the chamber. For the in vivo experiments, the animals ($n = 5$ mice/group) were placed in an animal HBO chamber (RSI-B11; Reimers Systems, Lorton, VA) and were exposed to HBO at 2 ATA for 90 minutes. The untreated control mice were placed outside the hyperbaric chamber in the same room. The HBO session for the in vivo experiments began with a progressive increase in pressure for 15 minutes, followed by 60 minutes of continuous exposure to 100% oxygen at 2 ATA. After 60 minutes of exposure, the pressure in the chamber was slowly reduced over a 15-minute period. After the procedure, the animals were placed in single cages in the animal care room.

Virus preparation

Adenoviruses expressing full-length mouse HIF-1α (Adv-HIF) stabilized against degradation by two point mutations (P402A/P563A) or the control vector LacZ-GFP was cloned into an E1- and partially E3-deleted serotype 5 adenovirus plasmid (as described in Botusan et al.[22]). The viruses were determined to be free of microbiological contaminants, mycoplasma, and endotoxin. The recombinant Adv-HIF or Adv-LacZ-GFP viruses (10^{12} viral unit/mL in phosphate-buffered saline [PBS]) were injected (100 μL) into the wound area.

Animal experiments

Ten-week-old male diabetic (db/db) mice on the C57Bl/6J background (Charles Rivers, Jackson Labs, Bar Harbor, ME) were used. All experimental groups were age-, weight-, and blood glucose-matched. Wound healing experiments were performed as described previously.[22] Briefly, the mice were anesthetized with 3% isoflurane (Abbott, Des Plaines, IL). The hair on the backs of the mice was shaved using a shaver followed by a depilatory cream. After cleansing the skin with alcohol, two full-thickness wounds extending through the panniculus carnosus were made on the dorsum on each side of the midline using a 6-mm biopsy punch. The adenoviruses were injected intradermally around the wound edges on the day of wounding. HBO was performed every day starting 1 hour after wounding. Mice received buprenorphine (0.03 mg/kg) twice a day to relieve any potential pain and distress for the first 2 days and were placed in single cages after wounding.

Digital photographs were recorded at the day of surgery and every other day after wounding. A circular reference was placed alongside to permit correction for the distance between the camera and the animals. The wound area was calculated in pixels with ImageJ 1.32 software (National Institutes of Health, Bethesda, MD), corrected for the area of the reference circle and expressed as percentage of the original area

The experimental procedure was approved by the North Stockholm Ethical Committee for Care and Use of Laboratory Animals and by the Institutional Animal Care and Use Committee from the University of Miami.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Stockholm, Sweden). The cDNA was obtained by reverse-transcribing the total RNA with SuperScript III and First-Strand Synthesis SuperMix for quantitative real-time polymerase chain reaction (RT-PCR) according to the manufacturer's recommended protocol (Life Technologies). The primers for VEGFA, PBGD[22] and for PGK1, BNIP3 were selected from the Harvard University primer bank (http://pga.mgh.harvard.edu/primerbank/). Power SYBR green with ROX reference dye (Life Technologies) was used for preparing the reaction mix, and RT-PCR was performed in an Applied Biosystems 7300 PCR unit. The amplification consisted of a 2-minute incubation at 50 °C followed by 10 minutes at 95 °C and a two-step cycling protocol for 40 cycles (15 seconds at 94 °C, 30 seconds at 60 °C). The melting curve and standard curve analysis was performed using the program from Applied Biosystems. Normalization and analyses were carried out by the $\Delta\Delta CT$ method using PBGD as the internal reference.[23]

VEGFA: Forward, 5′ CTTGTTCAGAGCGGAGAAAGC 3′
VEGFA: Reverse, 5'ACATCTGCAAGTACGTTCGTT 3′
SDF-1: Forward, 5′ ATTCTCAACACTCCAAACTGTGC 3′
SDF-1: Reverse, 5′ CTTCAGCCGGGCTACAATCTG 3′
PBGD: Forward, 5′ ACTCTGCTTCGCTGCATTG 3′
PBGD: Reverse, 5′ AGTTGCCCATCTTTCATCACTG 3′
PGK1: Forward, 5′ GAACAAGGTTAAAGCCGAGC 3′
PGK1: Reverse, 5′ GTGGCAGATTGACTCCTACCA 3′
BNIP3: Forward, 5′ CAGGGCTCCTGGGTAGAACT 3′
BNIP3: Reverse, 5′ CTACTCCGTCCAGACTCATGC 3′

Immunoblot detection of HIF-1α

HDF cultured in 60-mm plates were treated with HBO (2.5ATA, 100% oxygen) for 1 hour and then placed in a

normal cell culture incubator. After different periods of time (0, 2, 4, and 6 hours), the cells were chilled on ice and collected in iced PBS. The pellet was obtained after centrifugation (4,000 r.p.m. at 4 °C) and then frozen in liquid nitrogen. The proteins were extracted and analyzed as described before.[21] Briefly, the cell pellet was suspended in 100 μL of radioimmunoprecipitation assay buffer (10 mmol/L HEPES [pH 7.9], 400 mmol/L NaCl, 0.1 mmol/L ethylenediaminetetraacetic acid, 5% [vol/vol] glycerol, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride) followed by centrifugation at 4 °C for 20 minutes at 20,000 r.p.m. Protein concentration was measured using Bradford's assay with bovine serum albumin standards. Fifty micrograms of protein was loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis and then blotted to a nitrocellulose filter and blocked overnight with 5% nonfat milk in Tween PBS (TPBS). The membrane was incubated with the first antibody, rabbit anti-HIF-1α (Novus Biologicals, Stockholm, Sweden) (1 : 500), or rabbit anti-beta-Actin (Neomarkers, Fremont, CA) (1 : 3000) in TPBS for 2 hours. After several washes with TPBS, the membrane was incubated for 1 hour with the secondary peroxidase-linked anti-rabbit immunoglobulin G (Amersham, Uppsala, Sweden) diluted 1 : 3000 in TPBS. After washing with TPBS, the proteins were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

^{3}H-thymidine incorporation assay

HIF+/+ and HIF–/– MEFs were plated at a density of 2×10^4 cells/well in a 6-well plate. Cells were starved overnight and then exposed for two successive 60-minute HBO sessions (as described). Twenty-four hours following the last HBO, 1 μCi/mL of ^{3}H-thymidine (PerkinElmer, Boston, MA) was added to each well. Four hours later, the cells were washed twice with cold PBS and then with cold 5% trichloroacetic acid followed by solubilization with 0.5 N NaOH. The solubilized cells (400 μL) were mixed with 4-mL scintillation liquid and counted in a beta counter (Packard BioScience, Downers Grove, IL). Results are expressed as a percentage of the control (normoxia exposed) cells.

Reporter gene assay

The transcriptional activity of HIF-1α was assayed using plasmid (pT81/HRE-luc) containing HREs from erythropoietin. HDFs at 60–80% confluence were co-transfected in 12-well plates with 500 ng of HRE plasmid and 25 ng of Renilla luciferase-encoding plasmid (for normalization) (Promega, Finnboda Varvsväg, Sweden) using Lipofectamine 2000 in Opti-MEM (Life Technologies) for 3 hours, after which the medium was changed to regular cell culture medium (DMEM with 10% FBS). After 12 hours, HDFs were exposed to HBO (100% oxygen, 2.5 ATA for 60 minutes), and the luciferase activity was determined 24 hours later, as described by the manufacturer (Promega). Reporter gene activity was standardized to Renilla activity, and the fold induction is expressed as the activity in normoxic conditions.

Statistical analysis

Differences between groups were computed using one-way or two-way repeated measures analysis of variance as appropriate, with the Bonferroni post hoc test. A $p < 0.05$ was considered to be statistically significant. The sample size was estimated from previous studies[22] to detect a 20% difference in the average wound healing, with an α of 0.05 and β of 0.20.

RESULTS

HBO stabilizes and activates HIF-1

Immediately after exposure to HBO, HIF-1α is destabilized in HDFs, but it stabilizes starting at 4 hours after HBO treatment (Figure 1A). Vascular endothelial growth factor (VEGF), which is a classical target gene of HIF-1, is induced in SKRC7 cells that lack functional pVHL, which suggests that HBO stabilizes HIF-1α through a pVHL-independent mechanism (Figure 1B).

We further investigated whether the changes in protein stability are followed by changes in HIF transactivation. Exposure of HDF to HBO produced an increase in the HRE-driven luciferase response, confirming the functional relevance of the observed HIF-1 induction (Figure 1C).

This relevance was also confirmed by increases in VEGF and SDF-1α in HDFs exposed to HBO, both of which are classical target genes of HIF-1 (Figure 1D).

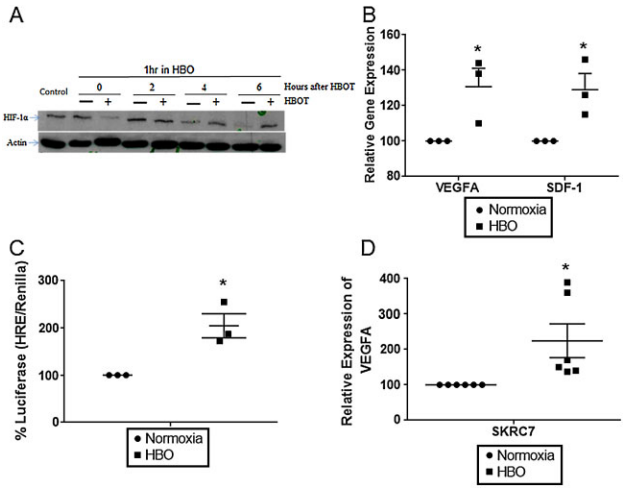

Figure 1. HBO stimulates HIF-1α. (A) Immunoblot detecting HIF-1α in HDFs exposed for different periods (0, 2, 4, and 6 hours) to HBO (pressure 2.5 ATA, 100% oxygen concentration). (B) HBO increases the relative expression levels of VEGF-A mRNA, even in SKRC7 cells that lack a functional pVHL (*$p < 0.05$). (C) Relative luciferase activity in the extract of HDFs transiently transfected with HRE-luciferase reporter plasmid was measured 24 hours after exposure to HBO (oxygen concentration 100%, pressure 2.5 ATA) or exposed to normal air for 60 minutes (*$p < 0.05$). (D) HBO increases VEFG-A and SDF-1 mRNA expression in HDFs at 24 hours after exposure (*$p < 0.05$).The bars represent the mean from five different experiments (*$p < 0.05$). ATA, atmospheres absolute; HBO, hyperbaric oxygen; HBOT, hyperbaric oxygen therapy; HIF-1α, hypoxia-inducible factor 1 alpha; HDF, human dermal fibroblast; HRE, hypoxia-responsive element; SDF-1, stromal-derived factor-1; VEGF, vascular endothelial growth factor.

HIF-1α mediates the HBO effect on the proliferation of human fibroblasts

We further investigated the functional relevance of the HIF-1α activation by HBO by examining the influence of HIF-1α on the HBO-induced proliferation of fibroblasts[6] using the loss-of-function genetic approach. MEFs that lack functional HIF-1α do not respond by increasing the proliferation rate to HBO as do wild-type MEFs, suggesting that HIF-1 induction plays a key role in HBO-dependent stimulation of cellular proliferation (Figure 2).

HIF-1 correction in diabetic wounds has a synergistic effect with HBO in diabetic animals

We performed adenovirus-mediated transfer of a constitutively stable form of HIF-1α (Adv-HIF-1α) in the wounds, which was able to increase the healing rate in db/db mice (Figure 3A). The combination of local induction of HIF-1α with HBO (70% ± 5% healing at day 7) had a better effect on wound healing compared with either therapy alone (HBO 20% ± 5% healing at day 7, Adv-HIF-1 40% ± 5% healing at day 7) ($p < 0.05$) (Figure 3B).

DISCUSSION

We have determined that HBO modulates HIF-1α, which plays a central pathogenic role in diabetic wound healing.

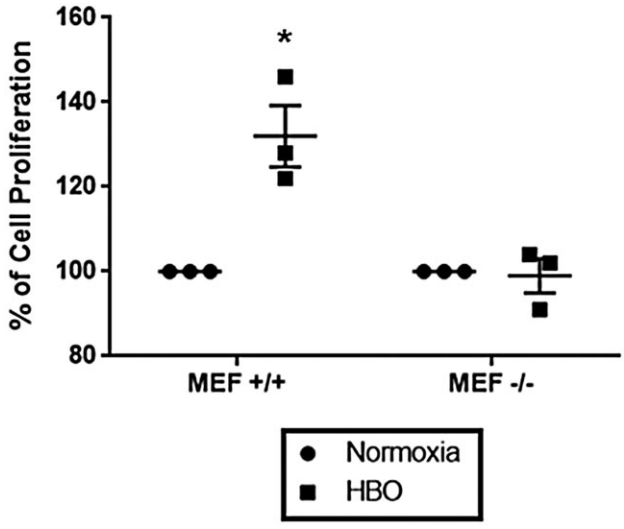

Figure 2. HBO stimulates fibroblast proliferation through an HIF-dependent mechanism. Wild-type MEFs (HIF+/+ MEFs) or those with non-functional HIF-1α (HIF–/– MEFs) were exposed to HBO for 60 minutes twice over a 24-hour interval, and cell proliferation was assessed 24 hours after the last exposure to HBO by ^{3}H-thymidine incorporation assay. Cell proliferation during HBO exposure is expressed as percent compared with the cells grown in normoxia, which are referred to as control. Experiments were performed three times. HBO, hyperbaric oxygen; HIF, hypoxia-inducible factor; MEF, mouse embryonic fibroblast. *p<0.05 compared with MEF +/+ exposed to normoxia.

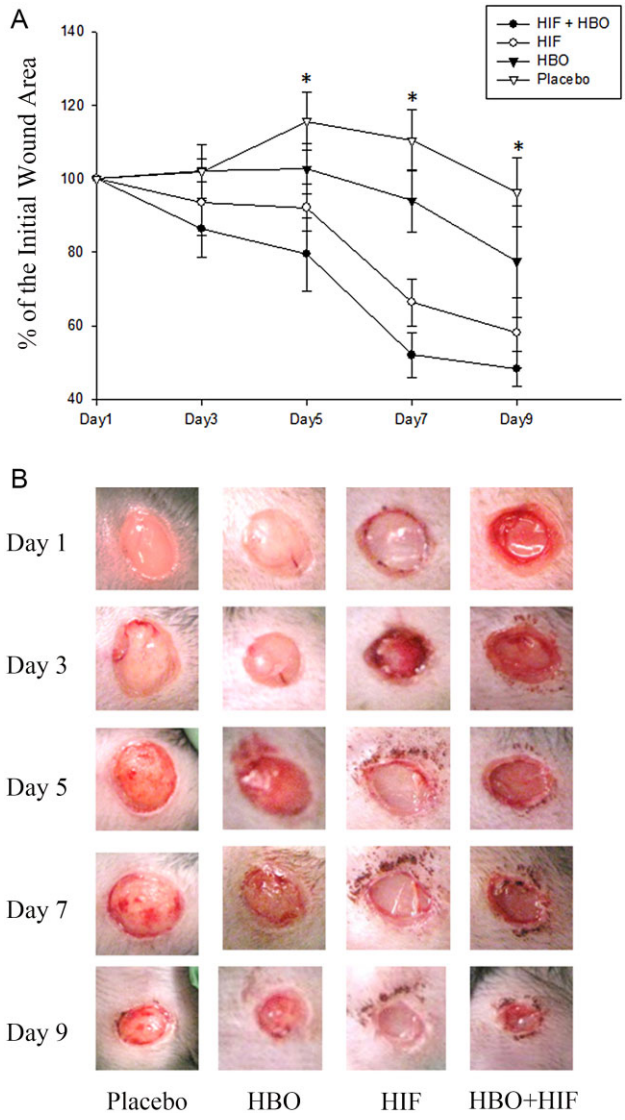

Figure 3. (A) Local stable HIF-1α has synergistic effects with HBO in promoting wound healing in diabetic mice. Diabetic mice (db/db) ($n = 5$) were treated every day for 90 minutes with HBO (2.5 ATA, 100% oxygen) (HBO), were locally injected on the day of wounding with adenovirus-transferred stable forms of HIF-1α (Adv-HIF) or empty virus (placebo), or were treated with the combination of locally injected adenovirus-transferred stable forms of HIF-1α (Adv-HIF) and HBO (2 ATA, 100% oxygen) (HBO + Adv-HIF) (values are means ± standard error of the mean, *$p < 0.05$, HBO + Adv-HIF vs. other treated and control groups). (B) Representative examples showing wound healing in db/db mice treated as above. HBO, hyperbaric oxygen; HIF, hypoxia-inducible factor.

Moreover, we demonstrated that local HIF-1 transfer in experimental diabetic wounds has synergistic effects with HBO, suggesting that HIF-1 induction may be a complementary therapeutic option for patients in need of HBO therapy.

HBO has a complex effect on HIF-1α by modulating both its stability and transactivation. The canonical regulation of HIF-1α consists of its stabilization against degradation in low oxygen levels through a VHL-dependent mechanism.[16] Therefore, at first glance, it is unexpected that high oxygen levels also have a stabilizing effect on HIF-1α. However, as expected, HIF-1 is undetectable immediately after high oxygen exposure (Figure 1A) and begins to stabilize only at later time points. This dynamic suggests that the stabilization of HIF-1α after HBO is not a direct effect of high oxygen levels but a mechanism that is activated later. Radical oxygen species (ROS) can be such mediators because their levels increase during exposure to HBO[24] and because they have a direct stimulatory effect on HIF-1α stability and function (recently reviewed in Chandel's study[25]). However, ROS modulate HIF levels through PHD, which implies VHL mediation, which is not the case for HBO. Nevertheless, HSP90 is such a potential mediator because it protects HIF-1α from degradation via a PHD2/pVHL-independent pathway,[26] and HBO induces HSP90 interactions with its partners.[27]

Several other mechanisms known to be involved in a VHL-independent stabilization of HIF could also mediate the effects of HBO, such as receptor for activated C kinase 1 RACK1,[28] mouse double minute 2 Mdm2,[29] Jun activation domain-binding protein-1 (Jab1),[30] forkhead box O4 (FOXO4),[31] and glycogen synthase kinase 3 (GSK3).[30]

HIF-1 stabilization after HBO is followed by an increase in its transactivation, as shown by HRE reporter gene assay and by the induction of classical HIF target genes in HDFs. Even though the cell culture model has obvious limitations such as the lack of complex multicellular interactions, we believe that our observation in a well-controlled cell culture environment provides clean evidence for the mechanistic modulation of HIF-1 by HBO that has been highly discordantly reported until now in different in vivo scenarios.[32–35] Even in previous studies performed on isolated cells from animals or patients exposed to HBO, the biological relevance of HIF-1 modulation was unclear, as both activators (HIF-1α and HIF-2α) and inhibitors (HIF-3α) of HIF function were reported to be similarly regulated.[36,37]

HIF-1α has potential pleiotropic effects on cellular processes with relevance for wound healing, such as cellular proliferation and migration, angiogenesis, and recruitment of EPCs.[38]

We first chose to monitor the relevance of HIF-1 on the HBO-dependent increase in fibroblast proliferation as a pivotal cellular process activated in wound healing.[6] MEFs lacking functional HIF-1 do not increase their proliferation rate in response to HBO as do wild-type MEFs, confirming the central role of HIF as a mediator of the positive effect of HBO on such an important cellular process. It is interesting to note in this context that the hypoxia-induced cellular proliferation is also dependent on HIF-1,[39] suggesting that HIF is a common player for both hypoxia and HBO in promoting wound healing.

Another essential mechanism that contributes to wound healing is the recruitment of EPCs to the hypoxic milieu of wounds. This process is mediated by several cytokines, in which SDF-1α plays a central role. SDF-1α is directly induced by HIF-1α,[14] and both are deficient in diabetic wounds.[13,20] Therefore, it is not unexpected that the correction of local HIF levels in diabetic wounds through the injection of stable adenovirus-mediated HIF has a synergistic effect with HBO (Figure 3). A similar effect was reported when SDF-1 was added to the diabetic wounds.[13] Because HIF-1 modulates several other important cellular processes involved in wound healing that are independent of SDF-1, it is expected that activation of HIF-1 would be superior to the addition of SDF-1. Wound healing in mice differs from human wound healing mainly because wound contraction plays an important role in rodents. However, this effect is decreased in db/db mice secondarily to the opposing tension in the skin as a consequence of obesity. Moreover, the major wound defects recorded in diabetic wounds in patients were also identified in the wounds of db/db mice[40]

In conclusion, we provide evidence that HBO stabilizes and activates HIF-1, which contributes to increased cellular proliferation. Moreover, we demonstrated that local induction of HIF-1α can be a potential additional therapy to HBO. This finding is interesting because several inhibitors of PHDs have been developed for clinical use.

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Conflicts of Interest: None of the authors have any potential conflicts of interest.

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