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Comparison of the Hydroxylase Inhibitor DMOG and the Iron Chelator Deferoxamine in Diabetic and Aged Wound Healing

Dominik Duscher[1,2,3], **Michael Januszyk**[1], **Zeshaan N. Maan**[1], **Alexander J. Whittam**[1], **Michael S. Hu**[1], **Graham G. Walmsley**[1], **Yixiao Dong**[1], **Sacha M. Khong**[1], **Michael T. Longaker**[1], and **Geoffrey C. Gurtner**[1,^]

[1]Hagey Laboratory, Division of Plastic Surgery, Department of Surgery, Stanford University School of Medicine, Stanford, CA, USA

[2]Section of Plastic Surgery, Department of Surgery, Johannes Kepler University, Linz, Austria

[3]Department of Plastic Surgery and Hand Surgery, Technical University Munich, Munich, Germany

Abstract

A hallmark of diabetes mellitus is the breakdown of almost every reparative process in the human body leading to critical impairments of wound healing. Stabilization and activity of the transcription factor HIF-1α is impaired in diabetes, leading to deficits in new blood vessel formation in response to injury. Here we compare the effectiveness of two promising small molecule therapeutics, the hydroxylase inhibitor dimethyloxalylglycine (DMOG) and the iron chelator deferoxamine (DFO), for attenuating diabetes-associated deficits in cutaneous wound healing by enhancing HIF-1α activation. HIF-1α stabilization, phosphorylation, and transactivation were measured in murine fibroblasts cultured under normoxic or hypoxic and low glucose or high glucose conditions following treatment with DFO or DMOG. Additionally, diabetic wound healing and neovascularization was evaluated in db/db mice treated with topical solutions of either DFO or DMOG, and the efficacy of these molecules was also compared in aged mice. We show that DFO stabilizes HIF-1α expression and improves HIF-1α transactivity in hypoxic and hyperglycemic states *in vitro*, whereas DMOG's effects are significantly blunted under hyperglycemic hypoxic conditions. *In vivo*, both DMOG and DFO enhance wound healing and vascularity in aged mice, but only DFO universally augmented wound healing and neovascularization in the setting of both advanced age and diabetes. This first direct comparison of DFO and DMOG in the treatment of impaired wound healing suggest significant therapeutic potential for topical DFO treatment in ischemic and diabetic disease.

^**Correspondence to:** Geoffrey C. Gurtner, MD, FACS, Johnson and Johnson Distinguished Professor and Associate Chairman of Surgery, Stanford University School of Medicine, 257 Campus Drive, GK-201, Stanford, CA 94305-5148, Fax 650-724-9501, ggurtner@stanford.edu.

DISCLOSURES

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Keywords

Wound healing; neovascularization; ROS stress; diabetes mellitus; aging

INTRODUCTION

Maintenance of oxygen homeostasis is critical for the survival of multicellular organisms, as evidenced by the ischemic necrosis that results when appropriate oxygen tension is not maintained in tissue infarction. Poor oxygen tension regulation is also thought to play a role in hyperproliferative disorders such as macular degeneration and diabetic renal failure. Furthermore, many tumors are thought to arise and thrive secondary to defects in HIF-1α regulation and control (1). HIF is a heterodimeric protein complex composed of two proteins that belong to a family of transcriptional activators that contain both basic helix-loop-helix and Per-ARNT-Sim domains, HIF-1α and HIF-1β. Once the two subunits dimerize, the complex then binds with its co-activators p300/CBP which allow HIF to bind to DNA and act as a transcriptional regulator of a multitude of downstream hypoxia responsive genes. Increased HIF-1α activity leads to transcription of several critical angiogenic factors, including VEGF, nitric oxide synthase (NOS), and SDF-1 (1, 2).

HIF-1β is a constitutively expressed protein that plays a role in many biological processes; however, HIF-1α is specific to hypoxia regulation. HIF-1α undergoes significant post-translational modification, in particular hydroxylation and phosphorylation, which controls the stability and activity of the HIF heterodimer (3). Mitogen-activated protein kinase (MAPK)-mediated phosphorylation of HIF-1α occurs after stabilization of the protein under hypoxic conditions. Phosphorylation of specific serine residues, Ser-641/643, on HIF-1α augments its transcriptional activity due to improved HIF-1β dimerization and subsequent DNA binding and HIF-1α nuclear accumulation (4, 5).

The post-translational hydroxylation of HIF-1α is under the control of a family of prolyl hydroxylases (PHD 1, 2, and 3) as well as an asparaginyl hydroxylase known as Factor Inhibiting HIF-1α (FIH). These hydroxylases belong to a family of iron-dependent dioxygenases which require iron, oxygen, and 2-oxaloglutarate (2-OG) as cofactors for the hydroxylation process. Therefore, these enzymes are inactive in the absence of oxygen, for example under hypoxic conditions; in the presence of iron chelators, such as deferoxamine; or in the presence of a 2-OG competitive inhibitor such as dimethyloxalylglycine (DMOG) (6, 7). The oxygen-dependent degradation domain (ODD) of HIF-1α contains two conserved proline residues, 564 and 402, that are hydroxylated by PHDs. Hydroxylation of HIF-1α occurs under normal oxygen tensions and provides a binding site for the von Hippel-Lindau protein (pVHL), which mediates the ubiquitination and proteasomal degradation of HIF-1α protein. Because the PHDs require oxygen as a co-activator, their activity is inhibited under hypoxic conditions, leading to decreased HIF-1α hydroxylation, decreased pVHL mediated ubiquitination and degradation, and increased HIF-1α protein stability. Loss or blockade of pVHL activity constitutively stabilizes HIF-1α protein under normoxic conditions (1). Additionally, hydroxylation of an asparagine residue (803) by FIH in the C-terminal transactivation domain of HIF-1α leads to inactivation of its transcriptional

activity by preventing the recruitment and binding of the HIF-1α coactivators p300/CBP (8–11). These biochemical reactions provide us certain therapeutic strategies to promote HIF-1α stabilization and transactivation by the inhibition of PHDs and FIH-1 activity.

Neovascularization, including vasculogenesis and angiogenesis, plays a critical role in the postnatal pathophysiology of ischemic disorders, tumor growth, wound healing, and diabetes (12). Angiogenesis, the sprouting of new blood vessels from pre-existing vessels, is the major mechanism underlying vascular remodeling in the adult. However, vasculogenesis has recently received considerable attention because the recruitment of bone marrow derived endothelial progenitor cells may lead to novel therapeutic approaches (13, 14). All forms of diabetes are characterized by chronic hyperglycemia-induced neovascular dysfunction, which contributes to impaired wound healing, impaired coronary collateral vessel development, and embryonic vasculopathy in pregnancies complicated by maternal diabetes (15). Poorly controlled diabetes leads to peripheral vascular disease with chronic ulcers and an increased rate of amputation (16, 17). Four major mechanisms have been hypothesized to cause the hyperglycemia induced vasculopathy seen in diabetic patients: increased polyol pathway flux, advanced glycosylation end-product (AGE) formation, protein kinase C (PKC) isoforms activation, and hexosamine pathway flux. The common link among these four pathogenic mechanisms is the overproduction of reactive oxygen species (ROS) by the mitochondrial electron-transport chain (18). Many studies have shown that the iron chelator deferoxamine (DFO) (19) scavenges oxygen free radical and can reduce the oxidative stress in diabetic hyperglycemia.

Our previous studies have shown that hyperglycemia decreases the production of VEGF by fibroblasts in diabetic patients and mice under hypoxic condition. Moreover, chronic hyperglycemia inhibits HIF-1α DNA binding and transcriptional activity by interfering with interactions with the co-factor p300 (20). This study seeks to further evaluate the molecular mechanisms underlying hyperglycemia induced HIF-1α dysfunction *in vitro* and *in vivo* and to explore potential therapeutic strategies employing DFO and DMOG in chronic hyperglycemia.

MATERIALS AND METHODS

Cell Culture

Mouse embryonic fibroblasts (MEFs) were generously provided by Dr. Karl Sylvester (Stanford University School of Medicine). All cells were cultured in DMEM (Invitrogen Corporation, Carlsbad, CA), supplemented with 10% FBS and 1% penicillin-streptomycin and were used for experimentation after passaged twice. For low glucose culture, DMEM with 5 mM D-glucose was used. For high glucose culture, 30 mM D-glucose was used. Chronic hyperglycemia was performed for 4 weeks using a high glucose DMEM medium changed every other day. DFO and DMOG concentrations (1 mM solutions) were used based on previously published data on in vitro and in vivo efficacy (20, 21). All hypoxia treatments were performed at 1% O2 for 16 hrs in a BioSpherix hypoxia work station (BioSpherix, Redfield, NY).

Western Blot

Cell nuclear protein extraction was performed with NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Next, 30μg of protein was separated on a 7.5% SDS-PAGE and immobilized on PVDF membrane (Millipore Corporation, Billerica, MA). The membranes were blotted with anti-HIF-1α (1:1000, Novus Biologicals Inc., Littleton, CO) and anti-β-actin antibody (1:5,000, Sigma) at 4°C overnight. Secondary antibodies were either anti-rabbit IgG (1:10,000, Amersham Biosciences Inc., Piscataway, NJ) or anti-mouse IgG (1:5000, Upstate, Lake Placid, NY) and were incubated at room temperature for 30 minutes. Blots were detected with the ECL chemiluminescent reagent (Amersham Biosciences Inc.) for 5 minutes, and exposed to BioMax films (Kodak, Rochester, NY) for 10 minutes.

HIF-1α Transactivity Reporter Assay

5HRE-Luc (a kind gift from Dr. Amato Giaccia, Stanford University School of Medicine) is a luciferase reporter driven by a hypoxia-inducible promoter containing five tandem repeats of the hypoxia-response element (HRE). pGL3 (Promega, Madison, WI) was used as an HRE luciferase control. The DNA plasmids were transfected into fibroblasts according to the manufacturer's instructions using the Lipofectamine Plus reagent (Invitrogen). A *Renilla* luciferase expression vector (Promega) was co-transfected to adjust for variation in transfection efficiency. The luciferase assays were performed using Dual-Luciferase reporter system reagents (Promega) in a Monolight 3010 luminometer (BD Biosciences Pharmingen, San Diego, CA), and each reporter assay was repeated at minimum in triplicate.

Mammalian Two-hybrid Assay

pVP16-C/H1 containing p300 C/H1 domain and pGal4-HIF-1α(776–826) including HIF-1α C-terminal activation domain were generous gifts from Dr. Eric Huang (University of Utah). A CheckMate® pG5luc, pBIND and pACT were purchased from Promega and used as controls. The same transient transfection reagents and luciferase assay were used as described above.

Hydrogen Peroxide Assay

A H_2O_2 assay (Amplex Red; Invitrogen) was used to determine the presence of H_2O_2 according to manufacturer's instruction. Briefly, working solutions were made fresh for each assay and added to wells to a final volume of 100 μL. Samples were incubated for 30 minutes in the dark in a 96-well black plate with clear well bottoms. Fluorescence readings were obtained in a fluorescence reader (PerkinElmer Inc., Wellesley, MA) with excitation at 485 nm and emission at 580 nm. Wells were counted in triplicate.

Animals

All experiments were performed using protocols approved by Stanford University Animal Care and Use Committee (IACUC) guidelines. Aged (21 months old, National Institute on Aging (Bethesda, MD)) and diabetic (12 weeks, BKS. CG-M+/+Lepr<db>/J; (Jackson Laboratory, Bar Harbor, ME)) C57BL/6J mice were used for *in vivo* wound healing

experiments. All experiments in accordance with Stanford University Institutional Animal Care and Use Committees.

In vivo Excisional Wound Model

To evaluate the effect of DFO and DMOG on murine wound healing, paired 6-mm full-thickness cutaneous wounds were created on the dorsa of mice as previously described (22). Each wound was held open by donut shaped silicone rings fastened with 6-0 nylon sutures to prevent wound contraction. Aged and diabetic mice respectively were randomized into three treatment groups: daily application of 10 ul of DFO or DMOG drip-on (1 mM solutions) and PBS vehicle control (n=4 per group). All wounds were covered with an occlusive dressing (Tegaderm, 3M, St. Paul, MN). Digital photographs were taken on day 0, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21. Wound area was measured using ImageJ software (NIH, Bethesda, MD). Upon complete healing, wounds were harvested and processed for neovascularization assessment via CD31 immunohistochemistry.

Statistical Analysis

Response of control and treatment in each experiment were compared using an unpaired Student's *t*-test. All error bars represent the standard error of the mean (SEM).

RESULTS

Hydroxylase inhibition with DMOG normalizes HIF-1α expression and phosphorylation in hypoxia

DMOG is known to inhibit FIH-1 and the prolyl hydroxylases through competitive inhibition of 2-oxoglutarate (2-OG) and thus may represent an attractive therapeutic agent to attenuate HIF-1α impairment in diabetes (23). Here we compared HIF-1α expression, phosphorylation, transcriptional activity, and interaction with p300 between mouse embryonic fibroblasts (MEFs) cultured with and without DMOG treatment. Consistent with its function as an inhibitor of PHDs, we found that DMOG significantly increased HIF-1α protein stability across all conditions (Figure 1A–B).

However, we previously showed that impaired activity, rather than stability, is the main mechanism underlying HIF dysfunction (20). Therefore in order to restore HIF function, DMOG needs to not only increase HIF expression but also its hypoxic transactivity under hyperglycemic conditions. As such, we examined the effects of DMOG on HIF-1α transactivity using a mammalian two –hybrid luciferase reporter system. As expected, DMOG treatment significantly increased luciferase activity in cells cultured under normal glucose conditions in normoxia, and this effect was also observed in hypoxia (Figure 1C). Interestingly, when DMOG was applied to cells cultured under high glucose conditions, normoxic HIF-1α/p300 binding increased significantly, approximating that of untreated cells in hypoxia. However, under hypoxic high glucose conditions DMOG treatment failed to increase HIF-1α binding above baseline.

Finally, to determine the effect of DMOG-mediated hydroxylase inhibition on HIF-1α's activity as a transcriptional regulator, MEFs treated with DMOG were analyzed using an

HRE luciferase assay as previously described (20). Similar to the mammalian two-hybrid results above, DMOG treatment significantly increased luciferase activity in cells cultured under normal glucose conditions in both normoxia and hypoxia, as well as high glucose conditions under normoxia (Figure 1D). However, under hypoxic high glucose conditions DMOG treatment failed to increase HRE activity above baseline. This suggests that while DMOG is able to partially reverse the hyperglycemia-induced HIF dysfunction, a full recovery to normoglycemic activity is not achieved. Furthermore, as the latter culture conditions (hypoxic, elevated glucose) most closely mirror those of ischemic diabetic wounds, this suggests that DMOG may not be an ideal candidate for diabetic wound healing.

Taken together, these data indicate that DMOG-induced inhibition of HIF-1α hydroxylation improves its biological function by increasing HIF-1α phosphorylation and improving its interaction with its co-activator p300; however, these effects are significantly reduced under hypoxic, hyperglycemic conditions.

Deferoxamine improves HIF-1α function under hypoxic and hyperglycemic conditions

Deferoxamine (DFO) is an iron chelator that stabilizes HIF-1α by scavenging oxygen free radicals and inhibiting HIF-1α hydroxylation through iron chelation (20). We have previously shown that DFO increases HIF-1α expression in both high glucose and normal glucose culture (20). Here we evaluated the effect of DFO treatment on HIF-1α transactivation and HIF-1α/p300 binding. Using an HRE luciferase reporter assay, we found that DFO significantly increased normoxic HIF transactivation in MEFs under both high glucose and normal glucose conditions (Figure 1E). Unlike with DMOG, we also found that DFO treatment significantly increased HIF transactivation under hypoxia for cells in high glucose, as well as normal glucose, culture. HIF-1α/p300 binding was assessed using a mammalian two-hybrid luciferase reporter system, and we found that DFO treatment significantly increased HIF-1α binding under all culture conditions, including hypoxic and high glucose (Figure 1F). These findings, in conjunction with our DMOG data above, suggest that DFO and DMOG impact HIF-1α activity via separate mechanisms, and that while the ability of DMOG to inhibit prolyl hydroxylase activity does improve HIF-1α protein expression it is not as effective as DFO in its attenuation of HIF-1α activity.

Since reactive oxygen species (ROS) are believed to be responsible for many of the complications in chronic hyperglycemia, we also evaluated whether DFO could reduce ROS generated by high glucose. Using an H_2O_2 assay we found that DFO significantly attenuated the hyperglycemia-associated increase in ROS levels under hypoxic high glucose conditions, normalizing H_2O_2 levels to that of cells cultured under normal glucose conditions (Figure 1G). These data are consistent with DFO's proposed mechanism of improving HIF-1α biological function through scavenging oxygen free radicals.

Deferoxamine, but not DMOG, enhances wound healing in diabetic mice

Given our *in vitro* observations on the efficacy of DMOG and DFO at attenuating the diabetes-induced impairments in HIF-1α activity, we explored the therapeutic potential of these molecules in diabetic wound healing. Splinted excisional wounds were created on the dorsum of type 2 diabetic mice (db/db) as previously described (24), and mice received daily

treatment with either 10 ul of 1mM DMOG solution, 10 ul of 1mM DFO solution, or saline. Wounds were monitored and photographed every other day until closure (Figure 2A). DFO-treated wounds displayed significantly accelerated healing from day 7 onward and healed significantly faster than control-treated wounds (15 days vs 20 days, $p < 0.05$), whereas DMOG-treated wounds exhibited no improvement over control (18.7 days vs 20 days, $p = 0.39$) (Figure 2B–C). These results are consistent with our *in vitro* findings on the differential efficacy of DFO over DMOG in reversing the effects of chronic hyperglycemia and could be further confirmed on a histological level with an increase of neovascularization exclusive to the DFO treatment group (Figure 2D).

Both DMOG and DFO enhance wound healing in aged mice

To determine whether our observations above were specific to diabetes or were more broadly applicable to states of impaired wound healing, we compared the efficacy of DMOG and DFO in healing wounds from aged mice. Advanced age, like diabetes, is associated with elevated levels of prolyl hydroxylases, attenuated HIF-1α function, and impaired wound healing, making it an ideal candidate to evaluate DMOG and DFO. As above, splinted excisional wounds were created on the dorsum of 21 month old C57 black 6 mice, and mice received daily treatment with either 10 ul of 1mM DMOG solution, 10 ul of 1mM DFO solution, or saline. Wounds were monitored and photographed every other day until closure (Figure 3A). Both DFO-treated wounds and DMOG-treated wounds healed significantly faster than control-treated wounds (11.7 days vs 15 days, $p < 0.05$, and 12 days vs 15 days, $p < 0.05$, respectively) (Figure 3B–C). This could also be confirmed histologically via endothelial cell specific CD31 immunostaining, illustrating significantly higher levels of neovascularization in both treatment groups (Figure 3D). This suggests that the differential efficacy of DFO vs DMOG may be tied specifically to the pathophysiology of diabetes.

In summary, DFO stabilizes HIF-1α expression, improves HIF-1α activity as a transcriptional regulator, and facilitate the interaction between HIF-1α and p300 in hypoxic and hyperglycemic states *in vitro*. DMOG's effects, by contrast, are significantly blunted under hyperglycemic hypoxic conditions. *In vivo*, both DMOG and DFO enhanced wound healing in aged mice, stimulating angiogenesis, but only DFO universally augmented wound healing in the setting of both advanced age and diabetes. Our data suggest significant therapeutic potential for topical DFO treatment in ischemic and diabetic diseases.

DISCUSSION

Numerous studies have demonstrated that hyperglycemia and hypoxia play important roles in diabetic vasculopathy (25–28), neuropathy (29, 30), and retinopathy (31, 32). The common links among these studies are HIF-1α dysfunction and VEGF reduction leading to impaired healing in diabetic individuals. Others have attempted to improve diabetic wound healing by replacing single cytokines such as VEGF with only limited success. This suggests that defective diabetic neovascularization is associated with a more global HIF-1α defect rather than just its effect on VEGF production. However, the mechanisms of hyperglycemia induced HIF-1α dysfunction remain unclear and, to some extent, controversial. To have a significant clinical impact on correcting diabetic vasculopathy, a

better understanding of the HIF-1α defect must be elucidated. Here we further this knowledge and suggest a significant therapeutic method for achieving this goal.

Aging results in profound changes in metabolic and repair mechanisms throughout the entire organism, impairing its natural regenerative capacity (33, 34). Aging skin displays a phenotype characterized by atrophic dermal matrix with increased wrinkle formation and compromised wound healing (35, 36). In order to heal adequately, a dynamic sequence of interactions between several skin cell types, the extracellular matrix (ECM), and numerous cytokines is required (37). With advanced age, pathological changes of these processes occur and result in impaired tissue repair through insufficient neovascularization, stromal deposition, and epithelialization (38–42). On a molecular level aging is characterized by a destabilization of HIF-1α via enhanced activity of its degrading enzyme prolyl hydroxylase 2 (PHD-2) (39, 43) resulting in impaired release of growth factors. Recent studies from our group showed that fibroblast-specific deletion of HIF-1α critically impairs murine cutaneous neovascularization and wound healing and that short hairpin RNA silencing of PHD-2 improves it (44, 45). It could be further demonstrated that a loss of epidermal HIF-1α expression accelerates skin aging and affects reepithelisation (46). Additionally, evidence is accumulating that the dysfunctions associated with advanced age are linked to the effects of oxidative stress (47–49). Reactive oxygen species (ROS) accumulate in aged tissues because of a reduced antioxidant activity of aged cells leading to reduced neovascularization, impaired fibroblast proliferation and increased neutrophil recruitment (50). However, our findings showing a comparable efficacy of DMOG, which has no antioxidative effect (20), and DFO in aged wound healing, suggest that restoration of HIF-1α levels rather than reduction of ROS stress is the dominant mechanism accelerating regeneration of aged tissues.

DMOG is a 2-oxoglutarate (2-OG) analogue, and its function results from competitive inhibition of 2-OG which is attacked by ferric-superoxide species and generates CO2 and succinate during FIH-1 hydroxylation reactions (6). In diabetes, high glucose oxidation enriches the tricarboxylic acid cycle and increases metabolic byproducts including 2-oxoglutarate (18). Therefore we hypothesize that DMOG competitive inhibition is attenuated by excess endogenous 2-OG in chronic hyperglycemia, thus, requiring either pretreatment or increasing concentration.

Deferoxamine (DFO) has been the standard iron chelating therapy for transfusion related siderosis for 30 years, and has improved the life expectancy and quality of life for patients with thalassemia major (51, 52). Cell damage associated with iron overload arises from the accumulation of cytotoxic iron pools that promote production of reactive oxygen species (ROS) exceeding intrinsic cellular defense capacities (53). An intrinsic interaction exists between iron metabolism and glucose homeostasis which iron affects glucose metabolism, and glucose impinges on several iron metabolic pathways. Oxidative stress and inflammatory cytokines influence these relationships, amplifying and potentiating the initiated events (54). Iron depletion has been beneficial in endothelial dysfunction and coronary heart disease in diabetes (19, 55, 56). In our present studies, DFO dramatically reduced ROS induced by chronic hyperglycemia, which may account for the rescued

phenotype in diabetic ischemia. As expected, DFO improved HIF-1α function and angiogenesis both *in vitro* and *in vivo*.

During normal glucose and hypoxia conditions, HIF-1α is phosphorylated and facilitated to heterodimerize with HIF-1β. At the same time, low oxygen tension prevents HIF-1α from hydroxylation by FIH-1, so p300/CBP is easily recruited to activate the transcriptional machinery. However, chronic hyperglycemia increases intracellular reactive oxygen species, dephosphorylates HIF-1α and increases FIH-1 mediated hydroxylation. This results in HIF-1α dissociation from HIF-1β and p300 which impairs HIF-1α activity as a transcriptional regulator of hypoxia responsive genes leading to poor neovascularization and wound healing. Both DFO and DMOG inhibit FIH-1 activity in addition DFO decreases oxygen free radicals inferring a cytoprotective effect and DMOG increases HIF-1α phosphorylation which augments HIF-1α transcriptional function.

Limitations of our study include the lack of a complete dose response evaluation of DFO or DMOG administration. While we have used a previously validated dosage regimen for our in vitro and in vivo studies (20) it is still possible to obtain biased results because of insufficient dosing of the compounds. It could similarly be possible that the in vivo results are biased by the nature of the topical application, which has been performed as published previously (20) without an evaluation of different dosing amounts or frequencies. Also HIF-1α levels of treated wounds have not been investigated and therefore the verification of an accumulation of effective concentrations of the substances via this application route remained undetermined.

In aggregate, our findings suggest that DMOG can be effective at stabilizing HIF and increasing its phosphorylation, however, this effect did not persist under hyperglycemic hypoxic culture conditions, which most closely resemble that of diabetic ischemic neovascularization. As such, it was not surprising that DMOG failed to improve diabetic wound healing, even though it was effective in treating aged wounds. DFO, by contrast, was successfully able to increase HIF stabilization, phosphorylation, and interaction with its co-activator p300 under all culture conditions, including hyperglycemic hypoxia, as well as enhance wound healing in both diabetic and aged animals. This suggests that although both molecules may have considerable therapeutic value in treating HIF impairments, DFO is differentially efficacious for the treatment of impairments in diabetic neovascularization. This corroborates recent preclinical studies utilizing DFO transdermal delivery effectively as a therapeutic for diabetic ulcers (57).

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ABBREVIATIONS AND ACRONYMS

2-OG 2-oxaglutarate

DFO	Deferoxamine
DMOG	Dimethyloxalylglycine
FIH	Factor inhibiting HIF-1α
HIF	Hypoxia-inducible factor
HRE	Hypoxia-response element
MEF	Mouse embryonic fibroblast
mRNA	Messenger RNA
PHD	Prolyl hydroxylase
pVHL	von Hippel-Lindau protein
ROS	Reactive oxygen species
SEM	Standard error of the mean

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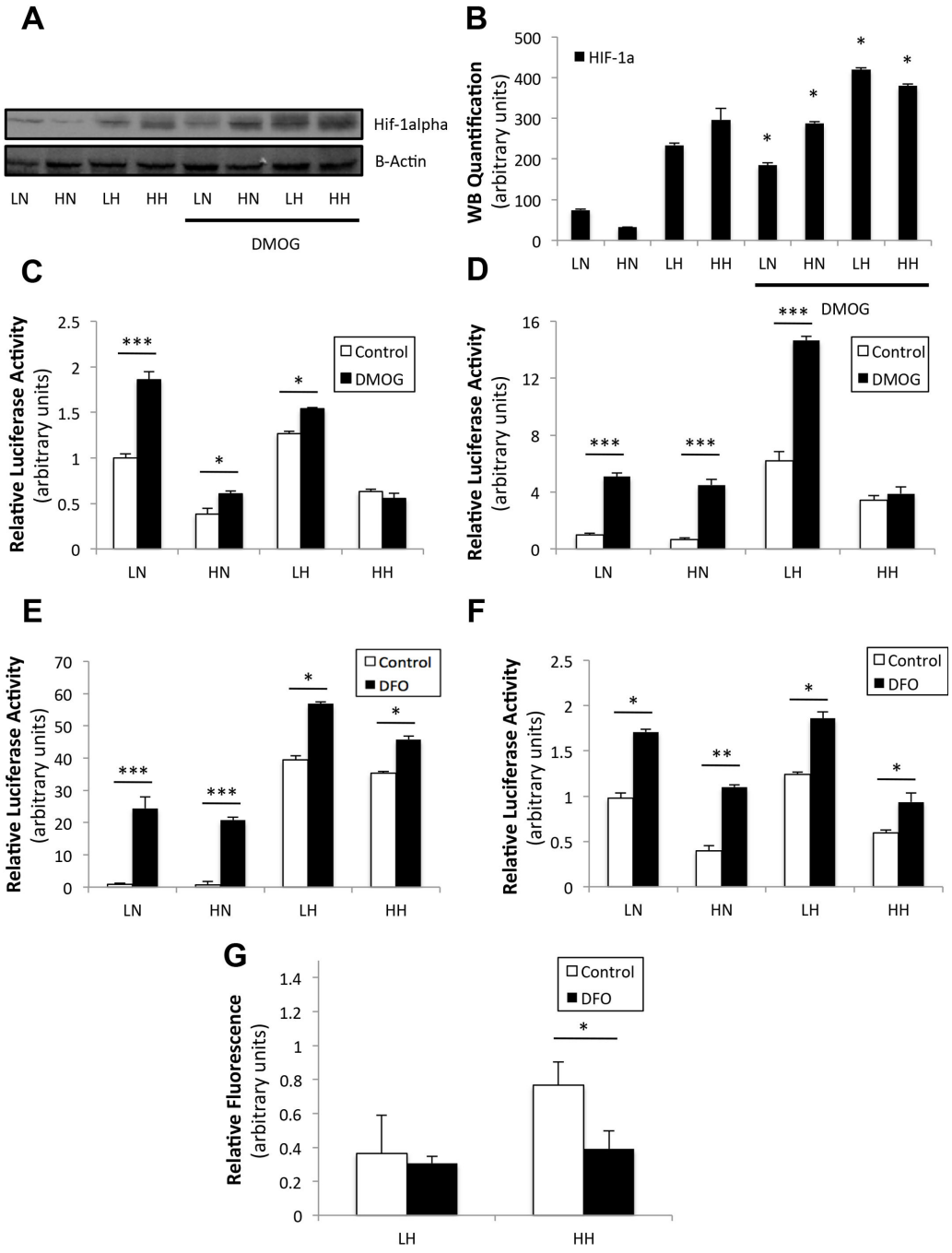

Figure 1. DFO increases HIF-1α activity and decreased ROS in MEFs in vitro while DMOG is unable to correct the high glucose effects

(A) HIF-1α western blots for MEFS with and without DMOG treatment cultured under low glucose normoxic (LN), high glucose normoxic (HN), low glucose hypoxic (LH), or high glucose hypoxic (HH) conditions. DMOG increases HIF-1α stabilization in all conditions. (B) Quantification of western blots. (C) Mammalian two hybrid luciferase activity measured in MEFs with or without DMOG treatment under various culture conditions. DMOG is unable to correct the high glucose effects. (D) HRE luciferase activity compared between MEFS with and without DMOG treatment under various culture conditions. DMOG is

unable to correct the high glucose effects. All measurements in panels C–D were normalized to that of control cells cultured under LN conditions. (E,F) Luciferase activity measured in MEFS with or without DFO treatment cultured under low glucose normoxic (LN), high glucose normoxic (HN), low glucose hypoxic (LH), or high glucose hypoxic (HH) conditions. HRE luciferase activity measured in MEFs is significantly increased with DFO treatment under all culture conditions. Mammalian two-hybrid luciferase activity MEFs is significantly increased with DFO treatment under all culture conditions. (G) The fluorescence measurements at 485 nm (excitation) and 580 nm (emission) are detected using an H_2O_2 reporter assay. DFO significantly reduces ROS generated by high glucose. All measurements in panels A–B were normalized to that of control cells cultured under LN conditions. n=3. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. All data are means ± one SEM.

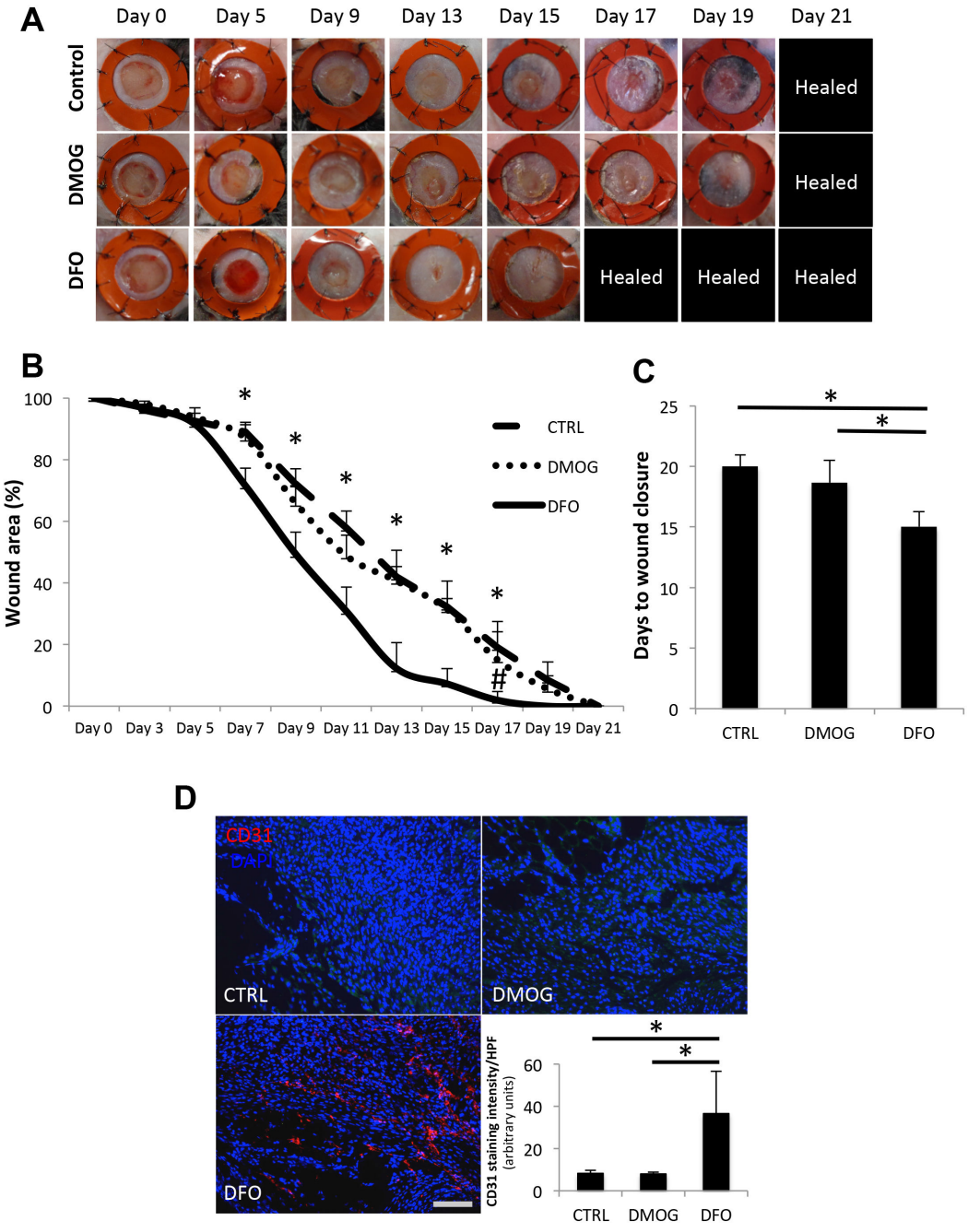

Figure 2. DFO but not DMOG enhances diabetic wound healing *in vivo*

(A,B) Gross appearance and area measurements of diabetic excisional murine wounds treated with DMOG and DFO. Application of DFO resulted in significantly accelerated wound healing from day seven on compared to wounds treated with DMOG or PBS control. (C) Application of DFO resulted in an accelerated time to wound closure compared to wounds treated with DMOG or PBS control. # indicates statistically significant differences in wound closure time between DFO versus DMOG and PBS control groups. (D) CD31 immunostaining and quantification were assessed upon complete healing. n=4. Scale bar=50μm. All data are means ± one SEM. * indicates $p \leq 0.05$. Scale bar = 100 μm.

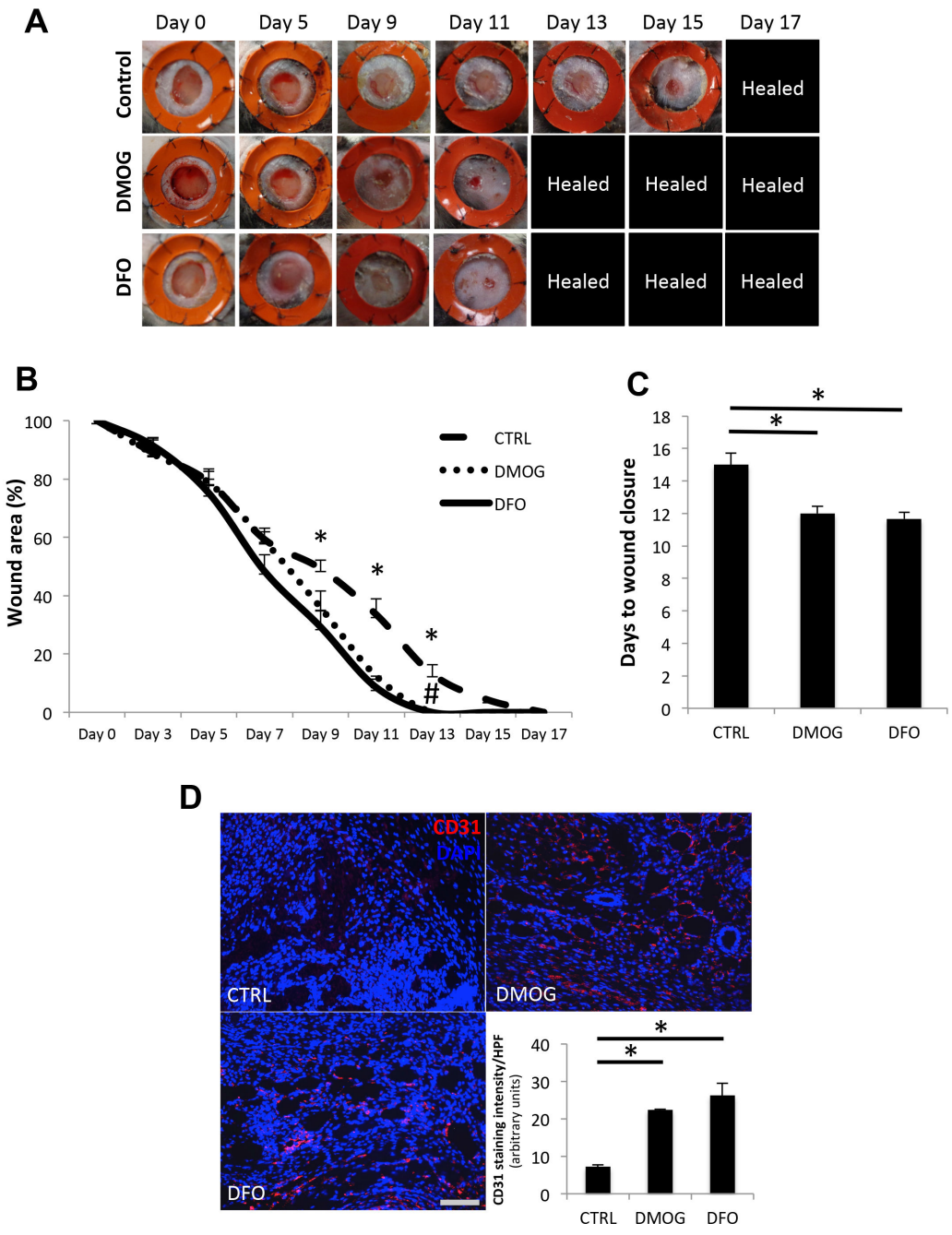

Figure 3. DFO and DMOG enhance aged wound healing *in vivo*

(A,B) Gross appearance and area measurements of aged excisional murine wounds treated with DFO and DMOG. Application of both DMOG and DFO resulted in significantly accelerated wound healing from day nine on compared to wounds treated with PBS control. (C) Application of DFO and DMOG resulted in an accelerated time to wound closure compared to wounds treated with PBS control. # indicates statistically significant differences in wound closure time between DFO and DMOG treatment versus the PBS control group.

(D) CD31 immunostaining and quantification were assessed upon complete healing. n=4. Scale bar=50μm. All data are means ± one SEM. * indicates $p \leq 0.05$. Scale bar = 100 μm.