Supporting Information

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SI Text

High glucose culture can also result in the development of HIF-1α-methylglyoxal adducts, with the most significant modification occurring at R23 (1). Formation of these adducts culminates in impaired HIF-1α-HIF-1β heterodimerization and a resultant decrease in HIF-1/DNA binding. Using the mammalian 2-hybrid system, we compared the relative influence of methylglyoxal modification of HIF-1α with the same alteration in p300, in contributing to the impaired interaction between these 2 proteins. Paralleling our previously noted results, high glucose culture reduced the association of HIF-1α with p300 by more than 50% (Fig. S5*A*). GLO1 overexpression completely reversed this effect. Substitution of the HIF-1α/R23Q mutant (where a point mutation of R23 converts this residue to Q and precludes methylglyoxal modification) in place of the wild-type HIF-1α protein did not improve the diminished association between HIF-1α and p300 in high glucose culture. In contrast, substitution of the p300/R354Q mutant in lieu of the wild-type p300 protein restored HIF-1α/p300 association to levels observed in low glucose culture. These data suggest that methylglyoxal modification of p300, not HIF-1α, underlies the impaired interaction between the 2 proteins in the high glucose environment.

Given our data demonstrating attenuated hypoxia-induced VEGF expression in the setting of high glucose culture, we used the mammalian 2-hybrid assay to evaluate the relative contributions of methylglyoxal modification of HIF-1α and p300 in producing this effect. High glucose culture of cells resulted in a greater than 50% decrease in the activity of a VEGF reporter plasmid, with this effect being abrogated by GLO1 overexpression (Fig. S5*B*). Use of either the HIF-1α/R23Q mutant or the p300/R354Q mutant resulted in partial improvement in VEGF reporter activity, although this correction fell short of the activity level observed in low glucose culture. Simultaneous use of both mutants, however, fully corrected reporter activity, suggesting that impairment of both HIF-1α-HIF1-β dimerization and HIF-1α-p300 association underlie the diabetes-induced defect in HIF-1 transcriptional activity.

SI Methods

Cell Culture. This study was approved by the Institutional Review Boards of the Stanford University School of Medicine and the Albert Einstein College of Medicine. Primary cultures of human fibroblasts were established from newborn human foreskins or from viable skin from diabetic and nondiabetic amputation specimens by collagenase digestion. C2C12 mouse myoblasts and MEFs were obtained from the American Type Culture Collection. All cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma and Gibco). Conditionally transformed HAECs were obtained from Dr. Anita Sumaga (Albert Einstein College of Medicine), and maintained in supplemented EBM-2 medium (Cambrex). Dermal fibroblasts from STZ-induced diabetic mice were cultured briefly in high glucose media before use in experiments. The same cells obtained from wild-type mice were cultured in low glucose. Human fibroblasts from amputation specimens were cultured in 5 mM D-glucose for $\approx$1 week and used at the first passage; all other primary cells were used after 4 weeks of continuous culture unless otherwise specified. For low glucose culture, DMEM with 5 mM D-glucose was used. For high glucose culture of HAECs, 30 mM D-glucose was used. D-glucose (25 mM) was used for high glucose culture of all other cell lines. DFO solution (100 μM) was used in indicated experiments. Cultures were exposed to hypoxia in a custom-designed incubator (X Vivo Hypoxia Chamber; Biospherix) set at 0.5% O_2 and 5% CO_2 or normoxia (21% O_2 and 5% CO_2); 1% O_2 was used for experiments involving HAECs.

VEGF ELISA. Quantikine murine VEGF ELISA kits (R&D Systems) were used according to the manufacturer's protocol.

Immunoprecipitation and Western Blotting. Cell lysates were precleared by preimmune mouse IgG (Santa Cruz Biotechnology) for 30 min with protein A-Sepharose 4B (Amersham Pharmacia Biotech) and incubated with the indicated antibodies for 2 h at 4 °C. Immune complexes were recovered with protein A-Sepharose 4B and washed 3 times with PBS plus 0.5% Tween-20 washing buffer. Total protein (50–80 μg) extracted with RIPA buffer was separated on a 7.5% or 10% polyacrylamide gel. Protein detection was performed with primary antibodies against p300, HIF-1α (Novus Biologicals), and β-actin (Thermo Fisher Scientific). Blots were simultaneously incubated with differentially labeled species-specific secondary antibodies after transfer to membranes [anti-rabbit IRDye 800CW (green) and anti-mouse Alexa 680 (red); LI-COR Biosciences]. Membranes were scanned and quantitated by the ODYSSEY Infrared Imaging System (LI-COR Biosciences). The monoclonal antibody to the major intracellular methylglyoxal-derived epitope, Nα-acetyl-Nδ (5-hydro-5-methyl)-4-imidazolone (methylglyoxal), was generated in this laboratory.

HIF-1 Transactivation Reporter Assays. The murine VEGF promoter (-2,000 to +460) was amplified by PCR of mouse fibroblast genomic DNA with introduction of restriction sites for *Kpn*I, *Mlu*I, or *Xho*I. Two reporter constructs (pVEGF-*kpnI*-luc and the VEGF reporter described in Fig. 3) were then created by cloning the full VEGF promoter into the pGL3-basic luciferase vector (Promega). Plasmid p2.1 was a gift from Dr. Gregg Semenza (The Johns Hopkins University School of Medicine). The 5$\times$ hypoxia response element construct was a gift from Dr. Amato Giaccia (Stanford University School of Medicine). Reporter plasmids were cotransfected with a constitutively expressed Renilla luciferase plasmid (pHRL-TK; Promega) using the Lipofectamine Plus reagent (Invitrogen). Luciferase activity was determined using the Dual Luciferase system (Promega) and normalized to Renilla luciferase activity.

Mammalian 2-Hybrid Assay. Expression plasmids for experiments evaluating the interaction between the HIF-1α-CAD and the p300-CH1 domain were a kind gift from Dr. L. Eric Huang (University of Utah, School of Medicine). The design of these plasmids has been described previously (2). Briefly, the Gal4-CAD expression plasmid was constructed by inserting HIF-1α-CAD (amino acid 776–826) into *Eco*RI/*Bam*HI-digested pCMX-G4(N). The VP16-CH1 expression plasmid was constructed by inserting the p300 CH1 domain into *Eco*RI/*Xba*I-digested p(His)VP16 (Clontech). Full-length HIF-1α and p300 were also subcloned into the Gal4-DBD-pM and Gal4-AD-pVP16 vectors, respectively. Specific deletions and point mutations for mapping of the methylglyoxal-responsive sites in p300 were prepared using PCR methods or the Site-Directed Mutagenesis kit from Promega. The luciferase reporter genes containing the Gal4-binding motif were also constructed as previously described, and all constructs were verified by DNA

sequencing (2). CheckMate pG5luc, pBIND, and pACT were purchased from Promega and used as controls. Transfection procedures and assessment of luciferase activity were as described above.

Real-Time Quantitative PCR. Total RNA from treated cells was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed by the SuperScript III First Strand Synthesis System (Invitrogen). Real-time quantitative PCR was run on a LightCycler (Roche) with the LightCycler FastStart DNA Master SYBR Green I kit (Roche). PCR was performed by denaturing at 95 °C for 7 min, followed by 45 cycles of denaturation at 95 °C, annealing at 60 °C, and extension at 72 °C for 10 s, respectively. cDNA (1 μL) was used for measurements, and the results were normalized to GAPDH.

In Vivo Murine Ischemia Model. All experiments were performed in accordance with the Stanford University Animal Care and Use Committee Guidelines. C57BL/6J mice (stock no. 000662; Jackson Laboratories), diabetic *db*/*db* mice (BKS.Cg-*m* +/+ Lepr^{db}, stock #000642; Jackson Laboratories), or STZ-induced diabetic C57BL/6J mice were subjected to a previously described murine model of cutaneous ischemia. Diabetes was induced in latter group following 5 consecutive daily injections of STZ (50 mg/kg; Sigma) in sodium citrate (3). Two and 4 weeks after the last injection, blood glucose levels were assessed using a glucometer (Roche Bioproducts). Mice with blood sugar levels <400 g/dL were excluded. Tissue oxygen tensions were measured in the ischemic flap with an OxyLite oxygen probe (Oxford Optronix) as previously described (4). To evaluate tissue VEGF levels, ischemic tissue was minced and homogenized in T-PER buffer (Pierce) containing protease inhibitors (Calbiochem). VEGF levels were quantified by ELISA as described above.

Peripheral Wound Model. Paired 6-mm full-thickness cutaneous wounds were made on dorsa of mice after depilation. As previously described (5), a donut-shaped 12 mm silicone splint (Grace Bio-Labs) was placed around the wounds and adhered to the skin with cyanoacrylate glue and interrupted 6–0 nylon sutures. Ten microliters 1,000 μM or 500 μM DFO (EMD Biosciences), or PBS vehicle, was introduced into the wound bed. Digital photographs of the wounds were taken every other day for 25 days. Wound area was quantified as a percent area of the original wound size using ImageJ software, with scaling normalized to the constant area of the splint.

At indicated time points, wounds were excised and snap-frozen or, alternatively, processed for H&E staining. Vascular density was detected on frozen sections using a CD31 monoclonal antibody (BD Biosciences) with a secondary Alexa-Fluor 488-linked anti-rat IgG antibody at 1:100 dilution. For quantification of CD31 positivity, wounds were analyzed under 200× magnification, and the number of positive 6 cells per high-power field (HPF) counted. All counts and observations were performed by a blinded observer.

Statistical Analyses. All data are expressed as mean ± SEM. Statistical analysis of results from the hypoxic induction of VEGF was carried out using factorial ANOVA, and results from the peripheral wound healing model were analyzed using a paired Student's *t*-test. Analysis for the remainder of the experiments was conducted by using analysis of variance and Tukey-Kramer statistical tests. $P < 0.05$ was considered statistically significant.

1. Ceradini DJ, et al. (2008) Decreasing intracellular superoxide corrects defective ischemia-induced new vessel formation in diabetic mice. *J Biol Chem* 283:10930–10938.
2. Gu J, Milligan J, Huang LE (2001) Molecular mechanism of hypoxia-inducible factor 1alpha -p300 interaction. A leucine-rich interface regulated by a single cysteine. *J Biol Chem* 276:3550–3554.
3. McEvoy RC, Andersson J, Sandler S, Hellerstrom C (1984) Multiple low-dose streptozotocin-induced diabetes in the mouse. Evidence for stimulation of a cytotoxic cellular immune response against an insulin-producing beta cell line. *J Clin Invest* 74:715–722.
4. Braun RD, Lanzen JL, Snyder SA, Dewhirst MW (2001) Comparison of tumor and normal tissue oxygen tension measurements using OxyLite or microelectrodes in rodents. *Am J Physiol Heart Circ Physiol* 280:H2533–H2544.
5. Galiano RD, Michaels J, Dobryansky M, Levine JP, Gurtner GC (2004) Quantitative and reproducible murine model of excisional wound healing. *Wound Repair Regen* 12:485–492.

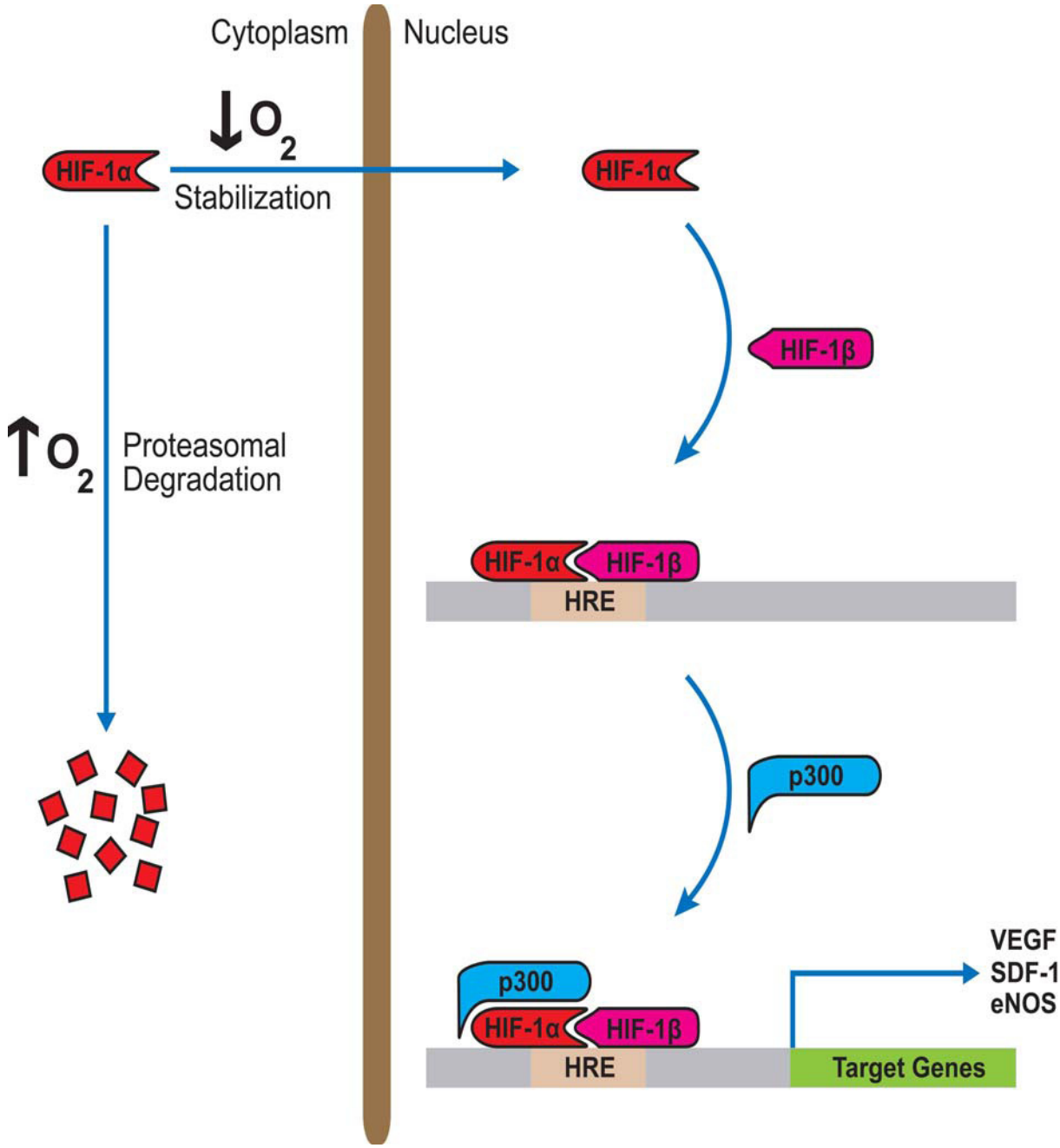

Fig. S1. The cellular response to hypoxia is mediated by HIF-1. Cellular hypoxia resulting from tissue ischemia leads to HIF-1α stabilization and translocation into the nucleus. HIF-1α dimerizes with HIF-1β and binds to a conserved hypoxia response element (HRE). Recruitment of the coactivator p300 results in transactivation of HIF-1-regulated genes such as VEGF, stromal cell-derived factor-1 (SDF-1), and endothelial nitric oxide synthase (eNOS). Normoxic conditions lead to proteasomal degradation of HIF-1α.

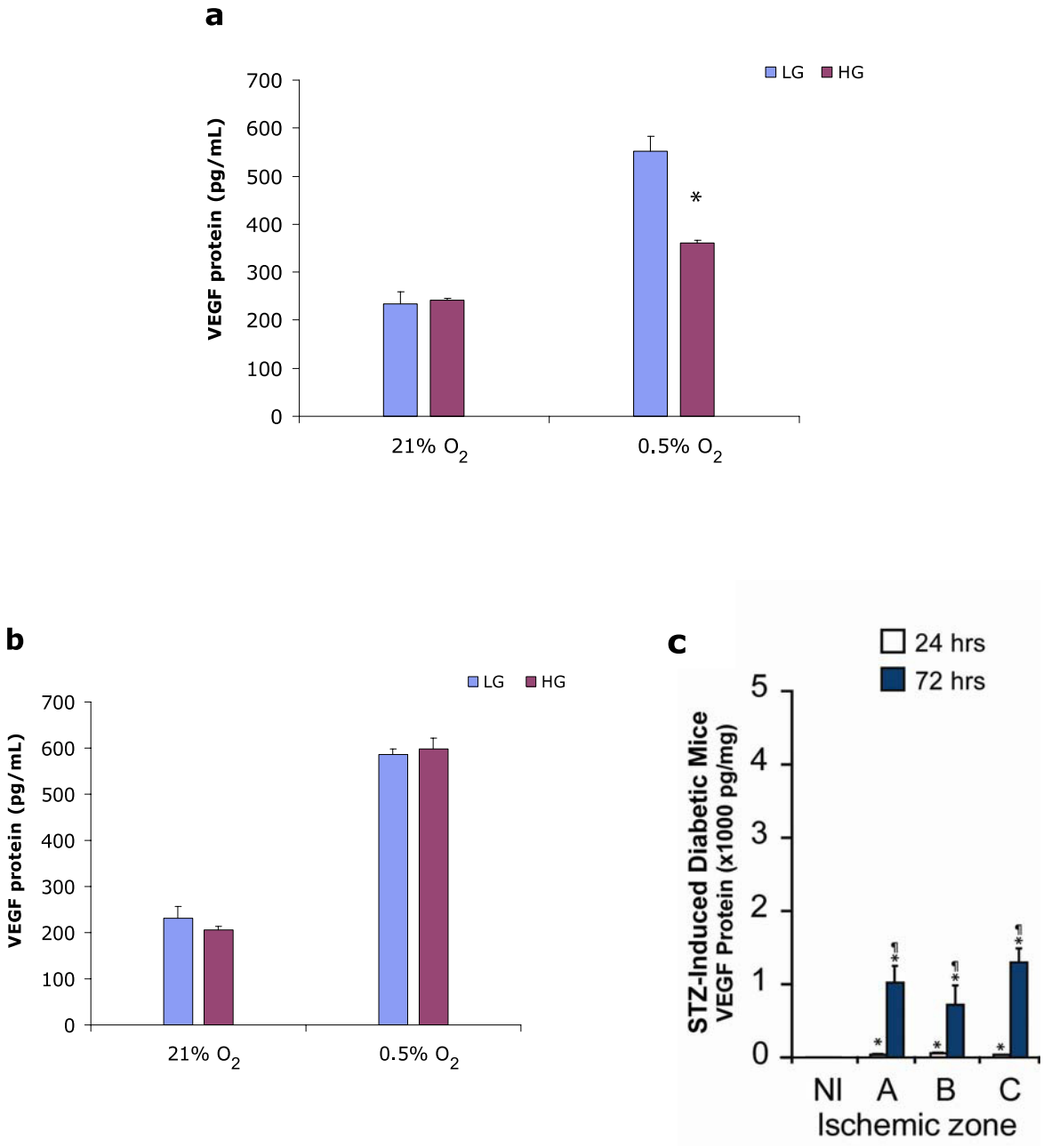

Fig. S2. Chronic high glucose exposure results in impaired VEGF expression. (*A*) C2C12 myoblasts grown chronically in LG or HG were placed in hypoxia or normoxia for 12 h, and levels of VEGF secretion were measured by ELISA. (*B*) Cells placed acutely (24 h) in LG or HG demonstrated no differences in hypoxia-induced VEGF production. (*C*) VEGF protein levels in ischemic skin tissue from STZ-induced diabetic mice measured 24 and 72 h after ischemic induction. All values represent mean ± SEM. *, $P < 0.05$ vs. LG (*A*) or baseline (*C*). ‡, $P < 0.05$ vs. zone A. ¶, $P < 0.05$ vs. 24 h. $n = 6$ for all panels. NI, normal skin.

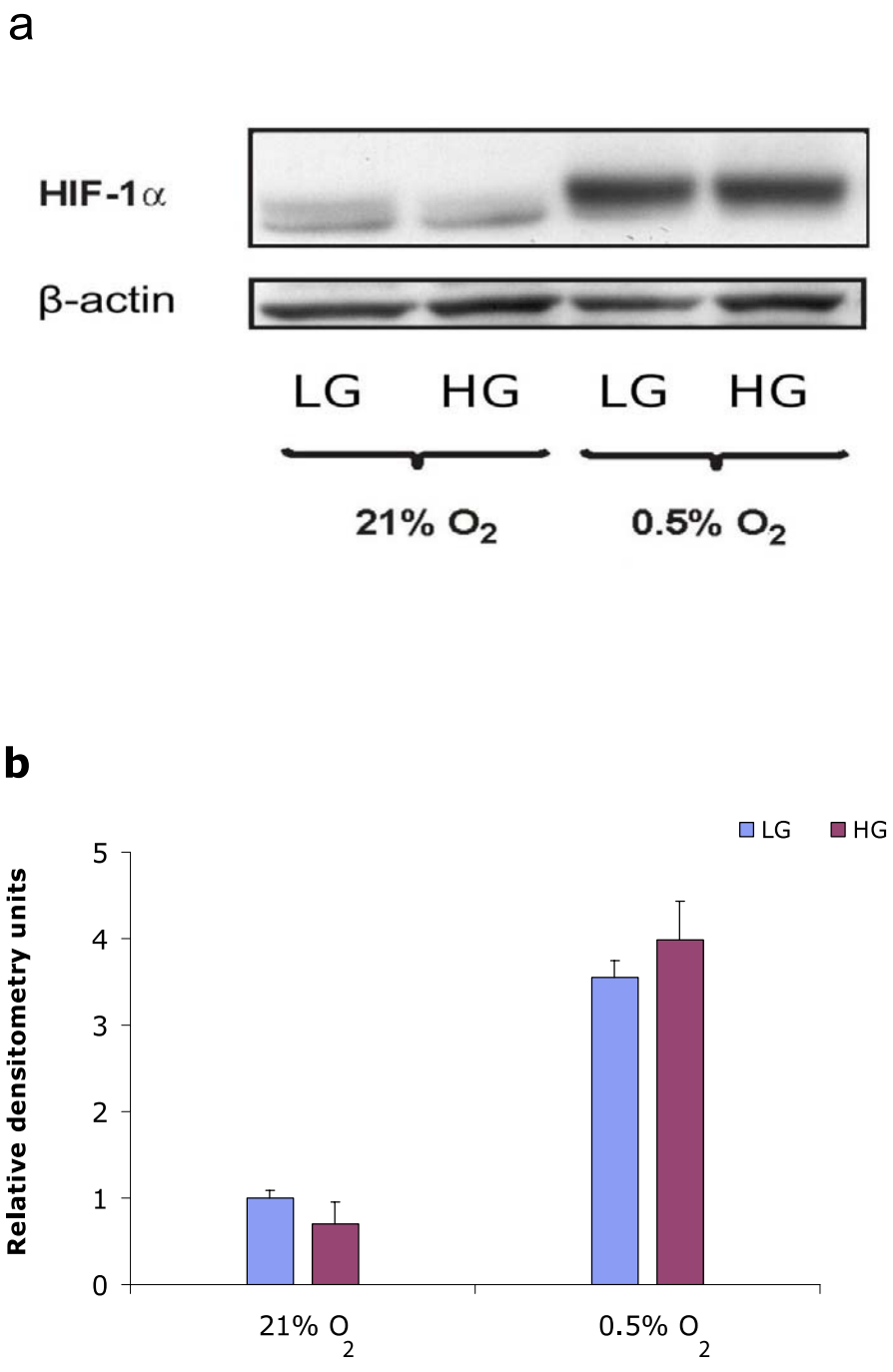

Fig. S3. HIF-1α protein stability is not impaired in hypoxic high glucose culture. (*A*) Representative western blot analysis of nuclear extracts from the culture conditions depicted. (*B*) Densitometric quantitation of HIF-1α protein levels did not demonstrate impaired expression in HG hypoxic conditions. Depicted values represent mean $\pm$ SEM. $n = 3$.

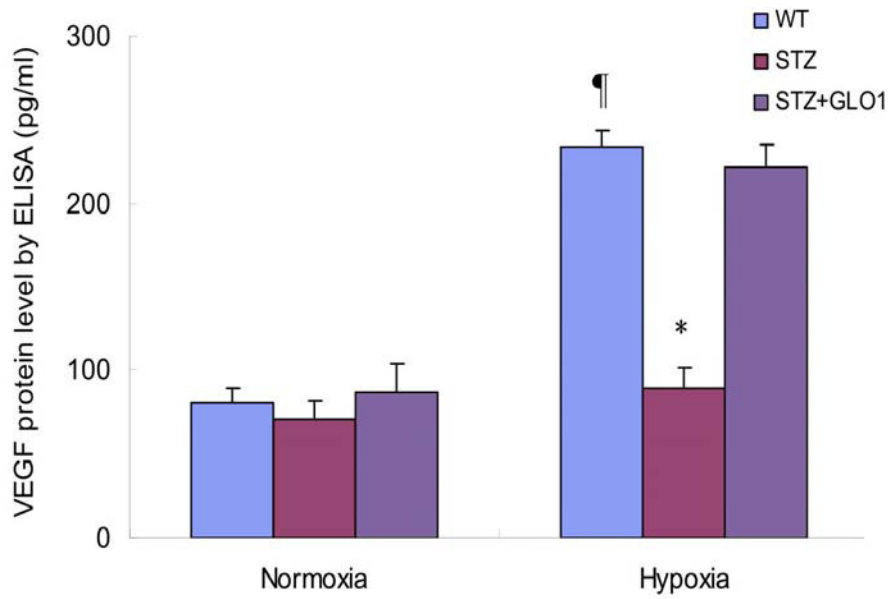

Fig. S4. Impaired hypoxia-induced VEGF expression in diabetic fibroblasts is corrected with GLO1 overexpression. Fibroblasts isolated from STZ-induced diabetic mice demonstrate decreased hypoxia-induced VEGF protein expression in comparison to wild-type (WT) mice. This is normalized by overexpression of GLO1. *, $P < 0.05$ vs. WT group; ¶, $P < 0.05$ vs. normoxia group. $n = 5$.

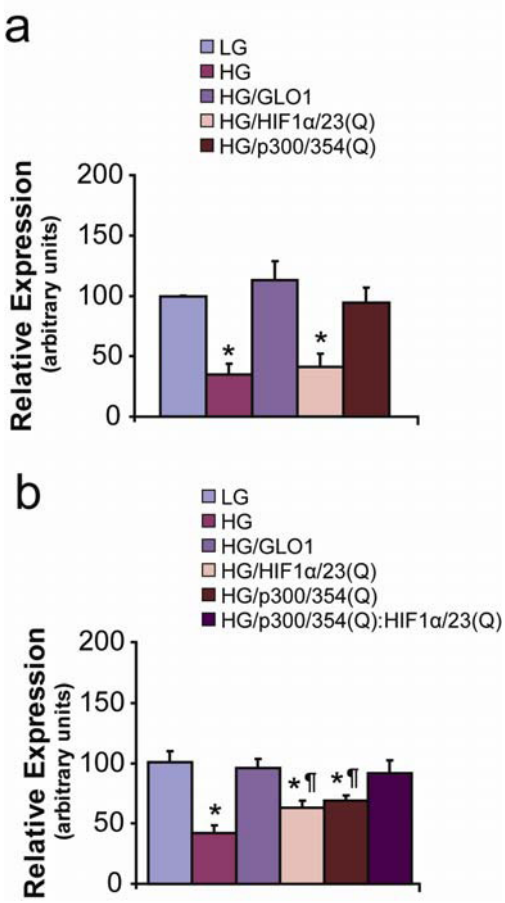

Fig. S5. Methylglyoxal modification of HIF-1α does not impair HIF-1α/p300 binding. (*A*) Mammalian 2-hybrid assay. HAECs were cotransfected with Gal4(DBD)-HIF-1α wild-type (bars 1–3) or single-mutant 23(Q) (bar 4), and Gal4(AD)-p300 wild-type (bars 1–4) or single-mutant 354(Q) (bar 5), plus pG5Luc (bars 1–5). (*B*) HAECs expressing the VEGF reporter were transfected with single-mutant p300/354(Q) alone, single-mutant HIF1α/23(Q) alone, or both mutants together. Cells were treated for 5 days in LG, HG, or HG/GLO1 as above; cells were then subjected to 18 h of hypoxia, and luciferase activity was measured. *, $P < 0.05$ vs. LG group. ¶, $P < 0.05$ vs. HG group. $n = 3$. AD, activation domain; DBD, DNA binding domain.

Table S1. Mouse oxygen level in ischemic skin segments

	Wild type mice		Diabetic mice	
Skin Segment	24 h	72 h	24 h	72 h
Baseline	38.2 (3.6)	40.6 (2.2)	37.0 (2.3)	42.1 (1.8)
A	31.4 (4.8)	29.9 (3.6)	24.9 (3.4)	23.6 (2.7)
B	16.8 (3.5)	7.1 (2.9)	11.4 (1.5)	5.8 (0.8)
C	9.8 (1.0)	2.3 (1.4)	7.5 (2.1)	2.7 (1.0)

Depicted are the mean O_2 values (in mm Hg) of ischemic skin segments from 3 normal and 3 diabetic mice at 24 and 72 h. The numbers listed in parentheses represent the SEM.