Hyperglycemia Regulates Hypoxia-Inducible Factor-1α Protein Stability and Function

Sergiu-Bogdan Catrina,[1] Kensaku Okamoto,[2] Teresa Pereira,[2] Kerstin Brismar,[1] and Lorenz Poellinger[2]

Hyperglycemia and hypoxia are suggested to play essential pathophysiological roles in the complications of diabetes, which may result from a defective response of the tissues to low oxygen tension. In this study, we show that in primary dermal fibroblasts and endothelial cells, hyperglycemia interferes with the function of hypoxia-inducible factor-1 (HIF-1), a transcription factor that is essential for adaptive responses of the cell to hypoxia. Experiments using proteasomal and prolyl hydroxylases inhibitors indicate that hyperglycemia inhibits hypoxia-induced stabilization of HIF-1α protein levels against degradation and suggest that mechanisms in addition to proline hydroxylation may be involved. This effect of hyperglycemia was dose dependent and correlates with a lower transcription activation potency of HIF-1α, as assessed by transient hypoxia-inducible reporter gene assay. Regulation of HIF-1α function by hyperglycemia could be mimicked by mannitol, suggesting hyperosmolarity as one critical parameter. The interference of hyperglycemia with hypoxia-dependent stabilization of HIF-1α protein levels was confirmed in vivo, where only very low levels of HIF-1α protein could be detected in diabetic wounds, as compared with chronic venous ulcers. In conclusion, our data demonstrate that hyperglycemia impairs hypoxia-dependent protection of HIF-1α against proteasomal degradation and suggest a mechanism by which diabetes interferes with cellular responses to hypoxia. *Diabetes* 53:3226–3232, 2004

Chronic complications of diabetes are a major health problem, and it has become a priority to characterize further their pathophysiological mechanisms to develop novel, rational therapeutic strategies. Even though prolonged exposure of the tissues to hyperglycemia seems to be the primary causative factor, some other factors may play a role as far as intensive blood glucose control reduces chronic complications but does not prevent them altogether (1,2). It has become increasingly evident that hypoxia plays an important role in all diabetes complications (3). In addition to deficient blood supply as a consequence of micro- and macrovascular disease, it has been postulated that hyperglycemia induces a pseudohypoxia state. This theory is based on the finding that high glucose concentrations induce a high $NADH^+/NAD^+$ ratio in cells even when the oxygen tension is normal (4).

Adaptive responses of cells to hypoxia are mediated by the hypoxia-inducible factor-1 (HIF-1), which is a heterodimeric transcription factor composed of two subunits, HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), both constitutively expressed in mammalian cells. Regulation of HIF-1 activity is critically dependent on the degradation of the HIF-1α subunit in normoxia. The molecular basis of its degradation is the O_2-dependent hydroxylation of at least one of the two proline residues (5,6) in the oxygen-dependent degradation domain of HIF-1α by specific prolyl 4-hydroxylases (PHDs) (7–9). In this form, HIF-1 α binds to the von Hippel-Lindau tumor suppressor protein, which acts as an E3 ubiquitin ligase and targets HIF-1α for proteasomal degradation (10,11). Under hypoxic conditions, HIF-1α is stabilized against degradation and upregulates a series of genes involved in angiogenesis, glycolytic energy metabolism, cell proliferation, and survival.

Even though hypoxia is the main regulator of HIF-1α, several other factors influence HIF-1α expression and function. Some of these factors are involved in diabetes pathogeny, e.g., insulin, IGF-I, IGF-II, and advanced glycosylation end products (AGE) (12–14). In this context, the effect of hyperglycemia on HIF-1α–mediated signaling has not been previously studied.

We therefore studied the effect of hyperglycemia on regulation of HIF-1α function by hypoxia. Human dermal fibroblasts (HDFs) and human dermal microvascular endothelial cells (HDMECs) that were grown in high glucose concentrations showed reduced levels of protein stabilization of HIF-1α and a lower transcription potency of the protein in response to hypoxia, as compared with cells that were grown in normal glucose concentrations. Osmotic challenge can be one mechanism by which hyperglycemia acts, because the effect of glucose could be mimicked by mannitol. In excellent agreement with these observations, we observed lower HIF-1α protein levels in diabetic wounds than in venous ulcers.

From the [1]Department of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; and the [2]Department of Cellular and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, Stockholm, Sweden.

Address correspondence and reprint requests to Sergiu-Bogdan Catrina, MD, PhD, Department of Molecular Medicine, Karolinska Hospital M1:O2, Stockholm, S-171776, Sweden. E-mail: sergiu-bogdan.catrina@molmed.ki.se.

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S.-B.C. and K.O. contributed equally to this work.

AGE, advanced glycosylation end product; ARNT, aryl hydrocarbon receptor nuclear translocator; DMEM, Dulbecco's modified Eagle's medium; EDHB, ethyl 3,4-dihydroxybenzoate; HDF, human dermal fibroblast; HDMEC, human dermal microvascular endothelial cell; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia response element; HSP, heat shock protein; PHD, prolyl 4-hydroxylase.

RESEARCH DESIGN AND METHODS

HDFs, HDMECs, and culture media were purchased from PromoCell (Heidelberg, Germany). All other cell culture reagents were from Gibco (Stockholm, Sweden). D-Glucose, MG 132, ethyl 3,4-dihydroxybenzoate (EDHB), and mannitol were purchased from Sigma (St. Louis, MO). The mouse monoclonal anti–HIF-1α (ab463) antibody was obtained from Abcam (Cambridge, U.K.). Rabbit polyclonal anti-ARNT antibodies have been described elsewhere (15). The mouse monoclonal anti-p53 antibody was from Oncogene Research Products (San Diego, CA), secondary peroxidase-linked anti-mouse IgG or anti–rabbit peroxidase–conjugated IgG was from Amersham (Uppsala, Sweden), and biotin-labeled anti-mouse IgG (H+L) was from Vector Laboratories (Burlingame, CA).

Cell culture. HDFs and HDMECs were maintained in a humidified atmosphere with 5% CO_2 at 37°C in commercially supplied fibroblast and endothelial cell growth media. Two weeks before the experiments, the cells were incubated in Dulbecco's modified Eagle's medium (DMEM; 5.5 mmol/l glucose) supplemented with 2 mmol/l L-glutamine, 100 IU/ml penicillin and streptomycin, and 10% heat-inactivated FCS. Only cells between passages 4 and 9 were used. P53−/− fibroblasts were grown under the same conditions.

RT-PCR. The expression levels of HIF-1 α RNA were evaluated by RT-PCR using β-actin as internal control. At the end of the incubation period, the cells were collected in PBS, and after centrifugation, 50 μl of RNA*later* (Ambion, Austin, TX) was added to the pellet, which was kept at −70°C until analysis. Total RNA was extracted using Micro-to-Midi Total RNA purification kit (Invitrogen, Paisley, U.K.) according to the manufacturer's instructions. After spectrophotometric quantification, reverse transcription was carried out using random primers and SuperScript II reverse transcriptase (Invitrogen). First-strand cDNA was amplified using HIF-1α specific primers 5′-CTGTGAT GAGGCTTACCATCAGC-3′ (left) and 5′-CTCGGCTAGTTAGGGTACACTTC-3′ (right) or β-actin specific primers using 5′-GACAGGATGCAGAAGGAGAT-3′ (left) and 5′- TTGCTGATCCACATCTGCTG-3′ (right). The amplification parameters were set for HIF-1α at 94°C for 2 min, 55°C for 18 s, 68°C for 1 min, 72°C for 1 min (26 cycles total) and for β-actin at 94.5°C for 5 min, 55°C for 18 s, and 72°C for 1 min (24 cycles total). Both reactions were in the linear range of amplification. The PCR products were electrophoresed on a 1.2% agarose gel that contained ethidium bromide.

Western blot analysis. At the end of the incubation period with different glucose/mannitol concentrations, a whole-cell protein extract was prepared as previously described (16). Briefly, the cells were collected in PBS, and after centrifugation, the cell pellet was frozen in liquid nitrogen. After thawing, the cell pellet was resuspended in 70 μl of extraction buffer (10 mmol/l HEPES [pH 7.9], 400 mmol/l NaCl, 0.1 mmol/l EDTA, 5% [vol/vol] glycerol, 1 mmol/l dithiothreitol, and 1 mmol/l phenylmethylsulfonyl fluoride) followed by centrifugation at 4°C for 30 min at maximum velocity. A total of 50 μg of total cellular proteins (measured by Bradford's assay with BSA standards) were blotted after SDS-PAGE to a nitrocellulose filter and blocked overnight with 5% nonfat milk in PBS. The membrane was incubated with anti–HIF-1α primary antibody (monoclonal antibody or anti–HIF-1α antiserum) in a 1:500 dilution in PBS that contained 1% nonfat milk for 2 h. After several washes, the membrane was incubated with the secondary peroxidase-conjugate antibody (1:750) diluted in PBS that contained 1% nonfat milk for 1.5 h. After extensive washing with PBS, the complexes were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Reporter gene assay. To assay the effect of glucose on the transcriptional activity of HIF-1α, we used a plasmid (pT81/HRE-luc) that contains three tandem copies of the erythropoietin hypoxia response element (HRE) in front of the herpes simplex thymidine kinase promoter and the luciferase gene (16). HDFs in six-well plates were transfected at 70–80% confluence with the reporter plasmid (0.5 μg/well), after changing the medium to Optimem (Life Science Technologies), using the FuGENE 6 transfection reagent (Roche Diagnostics, Bromma, Sweden) according to the manufacturer's instructions. After 6 h, the medium was changed to DMEM that contained various glucose/mannitol concentrations (5.5, 11, 25, and 30 mmol/l), and the cells were further cultured in triplicate samples under hypoxic (1% O_2) or normoxic conditions (21% O_2). After 48 h of incubation, luciferase activity was determined as described by the manufacturer (Promega Biotec, Madison, WI), and the reporter gene activity was standardized to protein content and expressed as -fold induction relative to the activity under normoxic, normoglycemic conditions.

Immunohistochemistry. Punch-skin biopsies were obtained from the margin of chronic diabetic or venous ulcers using local anesthesia when needed (Xylocaine with epinephrine; Astra-Zeneca, Södertälye, Sweden) and immediately deep frozen in liquid nitrogen (−70°C). Ethical approval for these experiments was received from the local ethics committee at Karolinska Hospital, and informed consent was obtained from each patient. The biopsies were cut by cryostat (8 μ) and directly fixed in acetone at 4°C for 10 min. After washing three times with PBS, endogenous peroxidase was blocked with H_2O_2 (0.3%) at room temperature for 15 min. The slides were incubated with the mouse monoclonal anti–HIF-1α antibody (2.95 μg/ml) diluted in PBS that contained 1% BSA and 0.1% Triton X-100 at 4°C overnight. On the second day, a biotin-labeled horse anti-mouse antibody was added for 30 min, and the sections were incubated with horseradish peroxidase ABC-complex (ABC Elite kit; Vector Laboratories) at room temperature for 45 min. The reaction was finally developed with DAB (Vector Laboratories), and the slides were stained with hematoxylin. For validation of the method, the same protocol was applied to HDFs that were grown on glass coverslips and incubated for 6 h in the hypoxic chamber (1% O_2) or kept in a normal incubator (21% O_2).

Each experiment was run at least three times, and representative figures are shown. The reporter assay results were analyzed by one-way ANOVA with a Tukey post hoc test.

RESULTS

Glucose inhibits in a dose-dependent manner hypoxia-induced upregulation of HIF-1α protein levels in HDF and HDMEC cells. We first investigated the effect of various glucose concentrations on HIF-1α expression at normoxia and hypoxia. After reaching confluence, HDFs or HMEDCs were cultured in DMEM with different glucose or mannitol concentrations (5.5, 11, 25, or 30 mmol/l). After 24 h, the medium was changed but maintained at the corresponding glucose/mannitol concentrations, and the cells were incubated either in a hypoxia chamber (1% O_2) or at normoxia (21% O_2) for 6 h. Whole-cell lysates were analyzed by immunoblotting. At normoxia, no HIF-1α protein could be detected by Western blot analysis of whole-cell extracts of either HDFs or HMEDCs, and exposure to high glucose did not have any effect on HIF-1α protein levels. In contrast, glucose impaired in a dose-dependent manner hypoxia-dependent accumulation of HIF-1α protein in HDFs (Fig. 1*A*). The reduction in hypoxia-dependent stabilization of HIF-1α protein levels was maximal at the highest dose of glucose tested (30 mmol/l glucose). A similar effect was also seen after incubation of HDFs with 30 mmol/l mannitol (Fig. 1*B*), suggesting that glucose-dependent inhibition of hypoxia-induced accumulation of HIF-1α could be mediated by an osmotic effect. In a similar manner, exposure of HMEDCs to either 30 mmol/l glucose or 30 mmol/l mannitol inhibited hypoxia-dependent stabilization of HIF-1α protein levels (Fig. 1*C*). Thus, exposure of both HDFs and HMEDCs to high glucose concentrations interferes with the process of hypoxia-regulated stabilization of HIF-1α.

Hyperglycemia interferes with protection from proteasome-mediated degradation of HIF-1α. To understand better the processes by which hyperglycemia interferes with HIF-1α upregulation in hypoxia, we investigated the influence of high glucose on HIF-1α RNA levels in normoxia and hypoxia. HDFs that were cultured in various glucose concentrations (5.5 and 30 mmol/l) for 24 h were exposed to different oxygen tensions (hypoxia 1% O_2 and normoxia 21% O_2) for 6 h after the medium was refreshed with corresponding glucose concentrations. Total RNA was analyzed by RT-PCR using β-actin as internal control. Hyperglycemia did not modify HIF-1α RNA levels in either hypoxia or normoxia, indicating that hyperglycemia does not regulate HIF-1α mRNA transcription (Fig. 2*A*).

Proteasome-mediated degradation at normoxia defines a central mechanism of regulation of HIF-1α function. We therefore studied the inhibitory effect of high glucose concentrations on HIF-1α protein levels in hypoxic HDFs

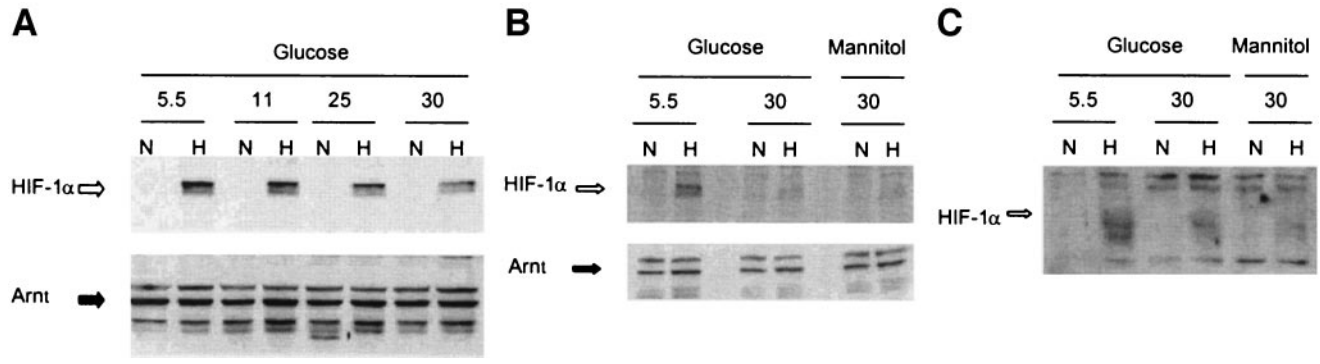

FIG. 1. Hyperglycemia impairs hypoxia-induced HIF-1α accumulation in HDFs and HDMECs. *A*: HDFs were grown for 24 h in various glucose concentrations and then exposed to either normoxia (N; 21% O_2) or hypoxia (H; 1% O_2) for 6 h. HDFs (*B*) and HDMECs (*C*) were grown as above, including mannitol as osmotic control. Whole-cell extracts were prepared and analyzed by Western blotting using anti–HIF-1α or anti-ARNT antibodies.

in the presence or absence of MG132, a specific inhibitor of proteasome activity. In the presence of MG132, the effect of high concentrations of glucose or mannitol on HIF-1α disappeared (Fig. 2*B*), suggesting that hyperglycemia interferes with hypoxia-dependent stabilization of HIF-1α against proteasomal degradation.

HIF-1α is targeted to proteasomal degradation after binding to von Hippel-Lindau, which functions as E3-ubiquitin-protein ligase. The interaction with von Hippel-Lindau requires the O_2-dependent hydroxylation of at least one of the two proline residues by specific PHD (7–9). We thus investigated whether the inhibitory effect of hyperglycemia on HIF-1α stabilization is dependent on the proline hydroxylation using EDHB, an inhibitor of PHD. We exposed the cells to EDHB at a dose above its reported *Ki* for all of the PHD (17). The PHD inhibitor induced HIF-1α stabilization in normoxia at the same level as hypoxia, confirming the efficiency of the chemical to block PHD in HDFs (Fig. 2*C*). It is not surprising to see that hypoxia intensifies the HIF-1α band in the presence of EDHB, because hypoxia may activate different mechanisms involved in protection of HIF-1α against degradation. In addition to a drop in proline hydroxylation, hypoxia modulates nuclear translocation, coactivator recruitment within the nucleus (16), HIF-1α acetylation (18), and so forth. Hyperglycemia was able to decrease the HIF-1α stabilization in the presence of EDHB in both normoxia and hypoxia, suggesting that other mechanisms, in addition to proline hydroxylation, may be involved in regulation of HIF-1α protein turnover in the presence of high glucose (Fig. 2*C*).

Hyperglycemia inhibits the hypoxia-induced HIF-1α stabilization over a prolonged period of time. We further studied the kinetics of the effect of hyperglycemia on hypoxia-induced upregulation of HIF-1α protein levels. In these experiments, HDFs, after reaching confluence, were grown in DMEM at either 5.5 (normal glucose concentration) or 30 mmol/l glucose. At different time points (6, 12, 18, 24, and 48 h) after exposure to these glucose concentrations, the cells were incubated at hypoxia (1% O_2) for 6 h before extraction of cellular proteins. As a reference, cells were incubated for 48 h with either normal or high glucose concentrations at normoxia (21% O_2). The inhibitory effect of the high glucose concentration on hypoxia-induced upregulation of HIF-1α could be observed as early as 6 h after of incubation and remained detectable thereafter (Fig. 3). During the time of the incubations used in this study, we did not observe any influence of glucose concentrations on HIF-1α protein levels under normoxic conditions.

Glucose-mediated inhibition of hypoxia-induced upregulation of HIF-1α is p53 independent. Given the reported effect of hyperglycemia on activation of p53 (19) and the link between p53 and regulation of HIF-1α stabil-

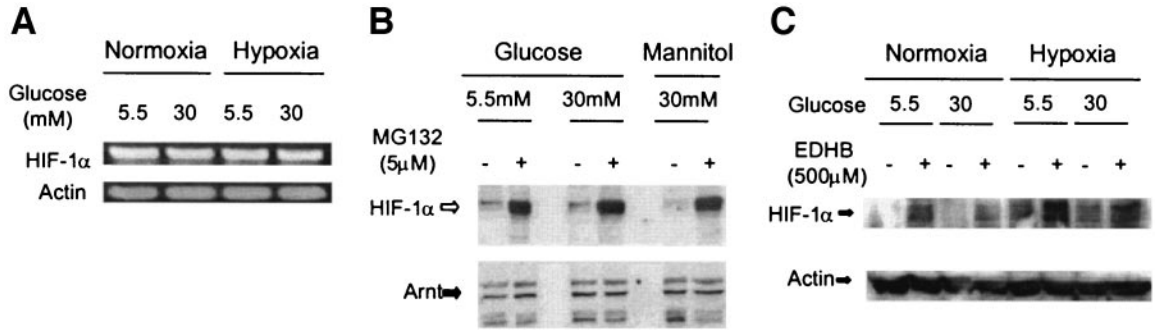

FIG. 2. Hyperglycemia interferes with proteasome-mediated degradation of HIF-1α. *A*: HDFs were incubated with different glucose concentrations for 24 h and exposed to either normoxia or hypoxia for 6 h. Total RNA was extracted and subjected to RT-PCR using specific primers for HIF-1α and β-actin (as internal control). The PCR products were electrophoresed on a 1.2% agarose gel that contained ethidium bromide. *B*: HDFs were incubated in hypoxia with various glucose and mannitol concentrations in the presence of MG132 or DMSO (as control) for 6 h. Whole-cell lysates were prepared and analyzed by Western blotting using anti–HIF-1α and anti-ARNT antibodies. *C*: HDFs were incubated with various glucose concentrations in the presence or absence of EDHB in either normoxia or hypoxia for 6 h. Whole-cell lysates were prepared and analyzed by Western blotting using anti–HIF-1α and anti-actin antibodies.

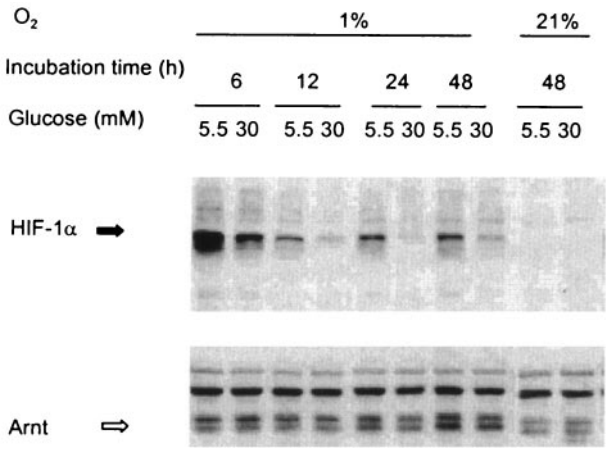

FIG. 3. Time course of the effect of hyperglycemia on hypoxia-induced stabilization of HIF-1α protein levels. HDFs were incubated for various periods of time with various glucose concentrations and exposed to normoxia or hypoxia for 6 h. Whole-cell extracts were analyzed by Western blotting using anti–HIF-1α and anti-ARNT antibodies.

ity at normoxia and hypoxia (reviewed in 20), we studied the effect of hyperglycemia on HIF-1α protein levels in p53−/− mouse fibroblasts. In agreement with the observations in HDFs and HDMECs, high glucose or mannitol concentrations inhibited hypoxia-induced upregulation of HIF-1α protein levels in p53−/− fibroblasts (Fig. 4) as well as in wild-type mouse fibroblasts (data not shown), indicating no role for p53 in mediating the effect of hyperglycemia on HIF-1α protein stability.

Hyperglycemia decreases HIF-1α function in hypoxic cells. Next we investigated whether the decrease of HIF-1α protein levels induced by hyperglycemia is followed by a decrease in the transcriptional activation function of HIF-1α. To study this, we transiently transfected HDFs with an HRE-driven luciferase reporter gene. This reporter gene generates a strong hypoxia-dependent activation response. Exposure of the cells to glucose concentrations >5.5 mmol/l, under hypoxic conditions,

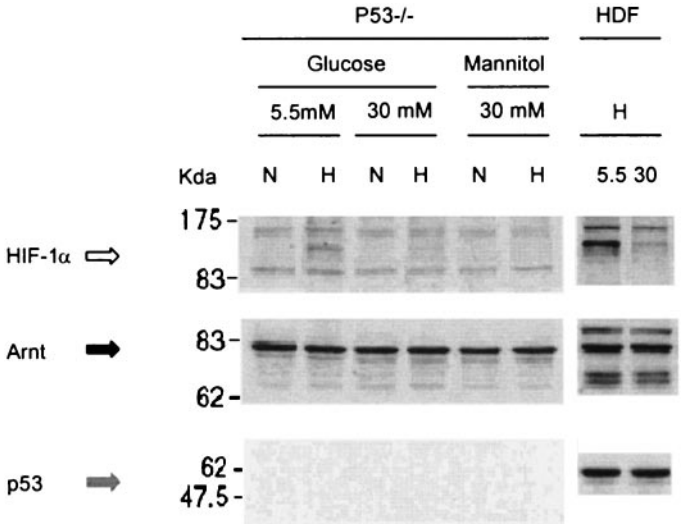

FIG. 4. Inhibition of hypoxia-induced stabilization of HIF-1α by hyperglycemia is not dependent on p53. p53−/− fibroblasts and HDFs were grown for 24 h in various glucose or mannitol concentrations and then exposed to normoxia or hypoxia for 6 h. Whole-cell protein extracts were analyzed by Western blotting using anti–HIF-1α, anti-ARNT, and anti-p53 antibodies. N, normoxia (21% O_2); H, hypoxia (1% O_2).

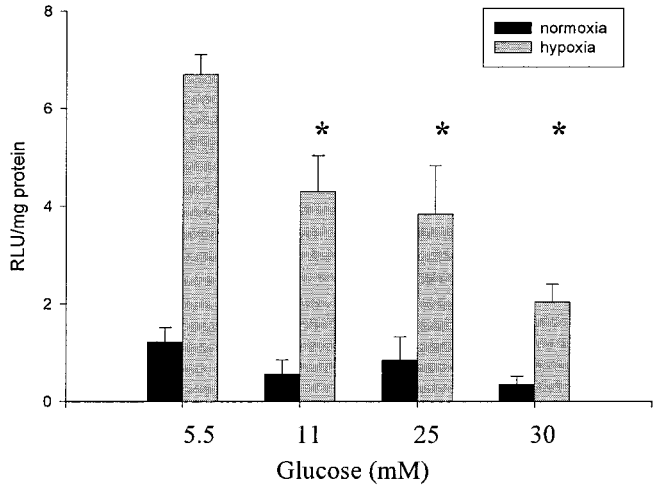

FIG. 5. Hyperglycemia decreases the functional activity of HIF-1α. HDFs were transiently transfected with an HRE-luciferase reporter construct and exposed to various glucose/mannitol concentrations and oxygen tensions for 48 h. Luciferase activity was standardized to protein content and expressed as fold induction relative to the activity under normoxic, normoglycemic conditions. The values represent means ± SE. Student's *t* test for paired values: *$P < 0.05$ between high glucose concentrations and normal glucose concentration at hypoxia.

produced in a dose-dependent manner a statistically significant decrease of the activation response (Fig. 5). In hypoxic cells, the glucose-induced effect on HIF-1α protein stability therefore correlates with a decrease in functional activity of HIF-1α. Also, in the reporter gene assay, the effect of hyperglycemia was mimicked by mannitol (data not shown), consistent with an osmotic effect on hypoxia-induced HIF-1α protein stability and function.

Diabetic chronic foot ulcers express lower levels of HIF-1α compared with chronic venous ulcer. To assess the in vivo significance of hyperglycemia-induced inhibition of HIF-1α protein stability at hypoxia, we studied the expression of HIF-1α by immunohistochemistry in biopsy materials of three diabetic chronic foot ulcers and two chronic venous ulcers. We compared these two chronic types of lesions because they had similar hypoxic environment but not the hyperglycemic condition. As a further reference, we studied by immunohistochemistry HIF-1α levels in normal HDFs at normoxia and hypoxia. Whereas a lack of staining for HIF-1α was observed in HDFs at normoxia (Fig. 6*A*), a strong nuclear staining of HIF-α was observed in hypoxic cells (Fig. 6*B*). Positive HIF-1α staining was also detected in both the cytoplasm and the nuclei of the majority of fibroblasts and some endothelial cells in venous ulcers (Fig. 6*C*). In contrast, diabetic ulcers showed only faint cytoplasmic staining in scattered fibroblasts (Fig. 6*D*), consistent with a role of hyperglycemia in regulation of HIF-α protein levels in hypoxic tissues.

DISCUSSION

We report here that hyperglycemia impairs hypoxia-dependent stabilization of HIF-1α against proteasomal degradation in primary HDFs and HDMECs. This observation suggests a mechanism of cross coupling between two of the most important determinants of the chronic complications in diabetes: hyperglycemia and hypoxia (rev. in 3). Both hyperglycemia and hypoxia have been suggested to be important causative factors for diabetic retinopathy

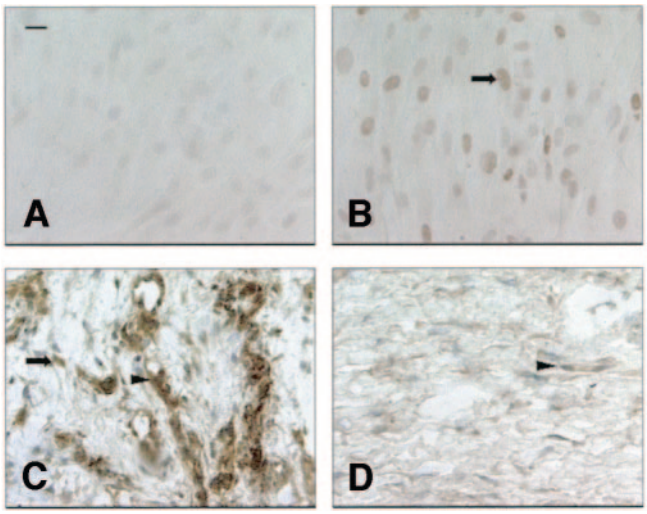

FIG. 6. Diabetic foot ulcers express lower levels of HIF-1α protein in comparison with chronic venous ulcers. Photographs illustrating brown (diaminobenzidine) immunoperoxidase staining for HIF-1α–positive cells counterstained with hematoxylin. *A*: Normoxic HDFs do not express HIF-1α. *B*: HDFs that were incubated for 6 h at hypoxia (1% O_2) express nuclear HIF-1α (➡). *C*: Venous ulcer biopsy material showing strong HIF-1α expression in endothelial and fibroblast cells, both in nuclei (➡) and in cytoplasm (➤). *D*: Diabetic ulcer biopsy with faint and scattered HIF-1α expression in fibroblast cytoplasm (➤). Bar = 15 μ. Magnification ×340.

(21), neuropathy (22), arteriosclerosis (23), kidney disease (24), and diabetic foot ulcers (25). Moreover, blood glucose was shown to be in linear relation with fatal outcome after an acute hypoxic challenge, i.e., acute myocardial infarction (26), suggesting a potential deleterious influence of hyperglycemia on the tissue's capacity to adapt to low oxygen tensions. Our data indeed prove that hyperglycemia interferes with the response of the cells to hypoxia, suggesting a new mechanism for development of chronic complications of diabetes. In light of our observation, it is worthwhile to mention the beneficial effect of deferoxamine (a powerful inducer of HIF-1α) on the dilation of cardiac vessels in diabetic patients or on other chronic complications of diabetes (recently reviewed in 27).

Here we also show for the first time that hyperglycemia interferes with HIF-1α stabilization even after chemical inhibition of PHD, which suggests that mechanisms in addition to proline hydroxylation may be involved. Several reports pointed out that p53 is able to destabilize HIF-1α by a von Hippel-Lindau–independent mechanism (rev. in 28). Moreover, p53 is induced by hyperglycemia (19), which makes us want to verify whether the effect of hyperglycemia on HIF-1α stabilization is mediated by p53. The persistence of the destabilizing effect of hyperglycemia on HIF-1α in fibroblasts that are p53 deficient powerfully suggests that p53 is not involved in the hyperglycemic-induced degradation of HIF-1α.

Another von Hippel-Lindau–independent proteasomal degradation mechanism for HIF-1α that involves heat shock proteins (HSPs) 90 and/or 70 was reported recently (29,30). Inhibition of these chaperones is followed by a decrease in HIF-1α protein stability and transcriptional activity in the same way as we observed after exposure of the cells to high glucose levels. It is therefore tempting to speculate that HSPs could be the mediators of the destabilizing effect of hyperglycemia on HIF-1α, especially if we take in account that HSP 70 is decreased in patients with diabetes or in experimental diabetes (rev. in 31).

In contrast to the effects of hyperglycemia at hypoxia, we could not detect any effect of hyperglycemia on HIF-1α protein levels in normoxic HDFs or HDMECs. This is in agreement with the observation made on normal retinal cells, where high glucose does not influence the normoxic levels of HIF-1α protein (32). However, the effect of glucose on HIF-1α protein levels seems to be cell dependent: in cancer cells, higher levels of HIF-1α are present at normal glucose concentrations than at low glucose concentrations (33). The effect of hyperglycemia on HIF-1α protein stabilization in hypoxic cells was mimicked by mannitol, suggesting osmolarity as one mechanism of action. However, mannitol at concentrations as high as 100 mmol/l had no effect on arsenite-induced upregulation of HIF-1α protein levels in ovarian cancer cell lines (34). The effect observed in the present study on HDFs and HDMECs could reside in a difference between (normal) primary cells and immortalized cells as well as in a possible difference between hypoxia and arsenite in their mechanisms of regulation of HIF-1α protein stability. In fact, we observed an opposite effect of hyperglycemia on HIF-1α hypoxic stabilization in some cancer cell lines (data not shown), which suggests that the difference resides in the phenotype of the cells.

Consistent with a negative effect of high glucose concentration on hypoxia-induced protein stabilization, hyperglycemia decreased the transcriptional activation function of HIF-1α, as assessed by reporter gene assays. Glucose has been reported to interfere with hypoxia-dependent activation of the liver pyruvate kinase promoter (35). Because of the high homology between the HRE and glucose responsive elements of this promoter, it has been proposed that the corresponding transcription factors HIF-1α and upstream stimulating factors, respectively, could compete for binding to either response element, thereby inducing a decrease of the cognate activation pathway (35). In the present study, the impaired stabilization of HIF-1α protein against proteasomal degradation correlates with the glucose-induced decrease in functional activity of HIF-1α in hypoxic cells, providing an alternative mechanism of regulation of HIF-1α function by glucose at hypoxia. In the case of cellular responses to hypoglycemia, a link between glucose concentrations and hypoxia signaling has also been observed. Embryonic cells deficient in HIF-1α are unable to upregulate several HIF-1 target genes at low glucose concentrations (36–38). However, the modulation by hypoglycemia of HIF-1α target genes seems to be dependent on the cell type varying from inhibition to stimulation (39). In our study, we show for the first time that high levels of glucose also influence HIF-1α protein levels and function in both fibroblasts and endothelial cells by negative interference with hypoxia-dependent stabilization of HIF-1α against proteasomal degradation.

The mechanisms by which hyperglycemia induces chronic complications have yet to be elucidated. Several biochemical pathways have emerged as pathophysiological mechanisms for glucose-induced diabetic complications: protein kinase C activation, increased polyol flux, increased AGE, and increased hexosamine pathway (rev.

in 40). Some of these pathways were investigated in relation to HIF-1α regulation, which helps us to focus our further investigations. Some members of the protein kinase C family of proteins modulate HIF-1α transactivation function but not the protein stabilization (41) or do not interfere with HIF-1α function at all (42). At normoxia, in a retinal epithelial cell line, AGE had a stimulatory effect on HIF-1α and its DNA binding (14). Activation of extracellular signal–regulated kinase has been suggested to mediate this effect, even though extracellular signal–regulated kinase was reported by others to be involved only in HIF-1α transactivation but not in HIF-1α stability (rev. in 43). Our experimental conditions were too short to generate AGE, which generally are produced after 1 week of incubation in high glucose concentration. Moreover, we were able to observe the same inhibitory effect of hyperglycemia on HIF-1α transcription activation also when HDFs were exposed for 2 weeks at high glucose levels (data not shown). The opposite effect between incubation with high glucose levels and AGE on HIF-1α levels could possibly reside in different regulation of HIF-1α in primary cells and cell lines. It is also possible that the effect observed by us, which can be mimicked by mannitol, is mediated by an osmotic mechanism different from that induced by AGE.

Hyperglycemia induced mitochondrial superoxide production, which has been proposed as the unifying mechanism for most other biochemical pathways activated by high glucose (44). It is therefore worthy to mention that reactive oxygen species were suggested for a long time in HIF-1α stabilization and transcription activity in hypoxia in a similar way as the effect we observed for hyperglycemia (rev. in 45). We have previously pointed out the importance of redox mechanisms on HIF-1α function (46), and we are currently investigating the relation among reactive oxygen species, hyperglycemia, and HIF-1α.

The importance of HIF-1α in fibroblast mobility and wound healing has recently been highlighted (47). Chronic venous ulcers share with diabetic ulcers the hypoxic environment (48) but not the exposure to high glucose levels. To test the in vivo relevance of our findings, we therefore analyzed HIF-1α protein levels in biopsy samples of diabetic and venous ulcers. We observed both nuclear and cytoplasmic expression of HIF-1α in fibroblasts of venous ulcer biopsy material. The cytoplasmic presence of HIF-1α could be due to enhanced protein stabilization and accumulation before nuclear translocation (49). In contrast, low levels of HIF-1α protein characterized the biopsy material from diabetic ulcers, generating faint cytoplasmic staining in scattered fibroblasts. These data suggest that inhibition of hypoxia-induced stabilization of HIF-1 protein by hyperglycemia in vitro also occurs in vivo. The clinical relevance of our findings in managing diabetic foot ulcers warrants further investigation.

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