High Glucose Blunts Vascular Endothelial Growth Factor Response to Hypoxia *via* the Oxidative Stress-Regulated Hypoxia-Inducible Factor/Hypoxia-Responsible Element Pathway

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Vascular endothelial growth factor (VEGF) is an important survival factor for endothelial cells in hypoxic environments. High glucose regulates certain aspects of VEGF expression in various cell types, including proximal tubular cells. Thus, ambient glucose levels may modulate the progression of chronic kidney disease, especially diabetic nephropathy. Immortalized rat proximal tubular cells (IRPTC) were cultured for 24 h under hypoxic conditions (1% O_2), with or without high D-glucose (25 mM), or with or without high L-glucose (25 mM). Controls included culture in normoxic conditions and normal D-glucose (5.5 mM). VEGF mRNA expression was assessed by real-time quantitative PCR, and VEGF protein in the supernatant was assessed by ELISA. Hypoxia increased VEGF expression. This response was significantly blunted by high D-glucose (1.98 ± 0.11- versus 2.65 ± 0.27-fold increase for VEGF mRNA expression, 252.8 ± 14.7 versus 324.0 ± 11.5 pg/10^5 cells for VEGF protein; $P < 0.05$ both) but not by high L-glucose. It is interesting that hydrogen peroxide also blunted this response, whereas α-tocopherol restored the VEGF response to hypoxia in the presence of high D-glucose. For determination of involvement of the hypoxia-inducible factor (HIF)/hypoxia-responsible element (HRE) pathway, IRPTC that were stably transfected with HRE-luciferase were cultured under the previous conditions. High D-glucose also reduced luciferase activity under hypoxia, whereas α-tocopherol restored activity. In vivo experiments using streptozotocin-induced diabetic rats confirmed that hyperglycemia blunted HIF-HRE pathway activation. Insulin treatment restored activation of the HIF-HRE pathway in streptozotocin-induced diabetic rats. In conclusion, high glucose blunts VEGF response to hypoxia in IRPTC. This effect is mediated by the oxidative stress–regulated HIF-HRE pathway.

Tubulointerstitial injury is closely associated with impairment of renal function and plays an important role in the progression of chronic kidney disease (CKD) (1,2). Hypoxia has been regarded as an important cause of these pathologic changes (3,4). However, severity of hypoxia is not the sole factor that determines the hypoxic injury.

Cells harbor many mechanisms with which they withstand hypoxic challenges; these mechanisms include increased expression of various protective molecules, such as vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1), and erythropoietin (EPO). These cellular hypoxic responses are regulated by the hypoxia-inducible factor (HIF)/hypoxia-responsible element (HRE) system, serving as a critical factor to determine hypoxic damages because induction of these genes has been shown to ameliorate renal ischemic injury (5).

VEGF is one of the most important survival factors for cells that are exposed to hypoxic insults. A potent stimulator of angiogenesis, it may help to restore the vascular supply to cells, thereby reducing hypoxia (6). In the kidney, VEGF that is produced by hypoxic proximal tubular cells can augment angiogenesis *in vitro* (7), and tubular VEGF expression correlates with the peritubular capillary density in the remnant kidney model (8). Moreover, VEGF seems to have the potential to mount a proliferative and antiapoptotic response in renal tubular epithelial cells (9). Although hypoxia is the major inducer of VEGF expression, VEGF production also is modulated by glucose concentrations, as well as several cytokines, growth factors, and hormones (10). We hypothesize that a high-glucose milieu may accelerate the progression of CKD by altering the protective VEGF response to hypoxia.

Although there are several reports of the effect of high glucose on VEGF expression, previous studies, to our knowledge,

focused on the effects of high glucose under normoxic conditions (7,11–13). The effect of high glucose on VEGF expression during hypoxia remains unclear. In this study, we performed studies with a proximal tubular cell line to clarify the effect of high glucose on hypoxia-induced VEGF expression.

Materials and Methods

Cell Culture and Experimental Design

Immortalized rat proximal tubular cells (IRPTC) (14) were cultured in normal-glucose (5.5 mM) DMEM (Nissui, Tokyo, Japan) buffered with 25 mM HEPES (Sigma-Aldrich, St. Louis, MO) at pH 7.4, supplemented with 5% FBS (JRH Biosciences, Lenexa, KS), 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.01 mM nonessential amino acids. The cells were grown at 37°C under a humidified atmosphere of 5% CO_2/95% air. IRPTC were synchronized for 24 h in serum-free 5.5-mM glucose DMEM at 80 to 90% confluence before the experiments.

High D-glucose culture medium (25 mM) was prepared by addition of 19.5 mM D-glucose into the medium, and 19.5 mM L-glucose (ICN Biomedicals, Aurora, OH) was added instead of D-glucose to produce high–L-glucose culture medium, serving as an osmolarity control. Hypoxia was induced by exposure to 1% O_2/5% CO_2 balanced with nitrogen in a multigas incubator (APM-30D; ASTEC, Fukuoka, Japan). All experiments were performed in the serum-free media to avoid the confounding effects of serum.

For investigation of the effect of high glucose on cellular responses to hypoxia, IRPTC were incubated for 24 h in (1) normal–D-glucose (5.5 mM) medium under normoxia as a control group, (2) high–D-glucose (25 mM) medium under normoxia, (3) normal–D-glucose medium under hypoxia (1% O_2), (4) high–D-glucose medium under hypoxia, or (5) high–L-glucose medium under hypoxia.

IRPTC also were cultured for 24 h under hypoxia in (1) normal–D-glucose medium that contained 100 μM hydrogen peroxide (Wako Pure Chemical Industries, Osaka, Japan) or (2) high–D-glucose medium that contained 50 μM α-tocopherol (Sigma-Aldrich) to explore further the mechanism of VEGF regulation by high D-glucose in hypoxic environment.

Experiments in HRE-Luciferase Stably Transfected IRPTC

The HRE-luciferase transgene vector (pHRE-LUC) was developed as previously reported (15). This transgene construct has high sensitivity and specificity reporter activity of the HIF-HRE pathway activation. The selectable marker vector pBK II-Neo was created by cutting the pcDNA3.1 (Invitrogen, Carlsbad, CA) with AseI, selecting the neomycin-resistant gene–containing fragments, and then cloning the fragment into pBluescript II at SmaI site.

IRPTC were co-transfected with 500 ng of pHRE-LUC and 25 ng of pBK II-Neo per 1×10^5 cells by using liposomes. The stable transfection of HRE-LUC transgene in IRPTC then was selected by culturing the transfected IRPTC in culture medium that contained 600 μg/ml geneticin.

The HRE-LUC stably transfected IRPTC (HRE-LUC IRPTC) were cultured for 24 h under the previously described conditions. The LUC activity of the cell lysate was determined using PicaGene firefly LUC assay kit (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions. The bioluminescent of the reaction was analyzed by Lumat 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

In Vivo Study: Streptozotocin-Induced Diabetes and Ischemia-Reperfusion Model in HRE-LUC Transgenic Rats

The previously established HRE-LUC transgenic rats (15) were randomly assigned to two groups, the control group and the streptozoto-

cin (STZ) group. The STZ rats received single intraperitoneal injection of STZ (Wako) 70 mg/kg on day 0. Hyperglycemia, defined by blood glucose >400 mg/dl, was confirmed in all STZ-injected rats 3 d after injection. All rats then were anesthetized by intraperitoneal ketamine and operated on using midline incision. Left renal arteries and veins were occluded for 45 min with microaneurysm clamps. At 15 min after clamp released, all rats were killed and the kidneys were removed for analysis. Right kidneys were used as nonischemic control in both groups.

In separate experiments, the transgenic rats were randomly assigned to three groups: The control group, the STZ-induced hyperglycemic group, and the STZ with insulin treatment group. The rats in the insulin treatment group received Humulin-N (Eli Lilly, Indianapolis, IN) subcutaneously, with a starting dose of 10 U/d followed by adjustment of the dose to keep blood glucose between 80 and 200 mg/dl. All rats then were killed and the kidneys were removed for analysis.

Total RNA Isolation and Real-Time Reverse Transcription–PCR

Total RNA was extracted from IRPTC or renal cortical tissue with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Reverse transcription was carried out by ImProm-II Reverse Transcription System (Promega, Madison, WI) using oligo(dT)$_{15}$ primer and 1 μg of isolated RNA.

One-twentieth (vol/vol) of cDNA product was subjected to real-time PCR (iCycler iQ; Bio-Rad Laboratories, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad) with the correspondence primers as follows: VEGF 5′-TTACTGCTGTACCTCCAC-3′, 5′-ACAGGACGGCTTGAA-GATA-3′; GLUT-1 5′-CAGTTCGGCTATAACACCGGTGTC-3′, 5′-ATAGCGGTGGTTCCATGTTT-3′; EPO, 5′-TACGTAGCCTCACT-TCACTGCTT-3′, 5′-GCAGAAAGTATCCGCTGTGAGTGTTC-3′; LUC 5′-TCATCGTTGACCGCCTGAAG-3′, 5′-CGTCTTTCCGTGCTCCAAAAC-3′; and β-actin 5′-CTTTCTACAATGAGCTGCGTG-3′, 5′-TCATGAGG-TAGTCTGTCAGG-3′ (5). After an initial hold of 15 min at 95°C, the cDNA samples were cycled 40 times at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Measurement of VEGF Protein

After 24 h, culture medium was collected and immediately centrifuged for 10 min at 10,000 rpm, and cell culture supernatant was stored at −80°C until analyzed. VEGF levels of cell culture supernatant were measured by quantitative sandwich ELISA using rat VEGF DuoSet (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Measurement of Reactive Carbonyl Compounds

The levels of three reactive carbonyl compounds (RCO)–glyoxal (GO), methylglyoxal (MGO), and 3-deoxyglucosone (3-DG)—in cell culture supernatant were measured by reverse-phase HPLC as described previously (16).

Statistical Analyses

Data were expressed in mean ± SEM. Unpaired t tests were performed to compare the mean between two groups. One-way ANOVA with post hoc analysis by the least significant difference method were used to determine the differences of mean among more than two groups. The significance level was set at 5% for all tests.

Results

High Glucose Blunted the VEGF Response to Hypoxia

VEGF mRNA expression in IRPTC was estimated by quantitative real-time PCR analysis (Figure 1A). VEGF mRNA ex-
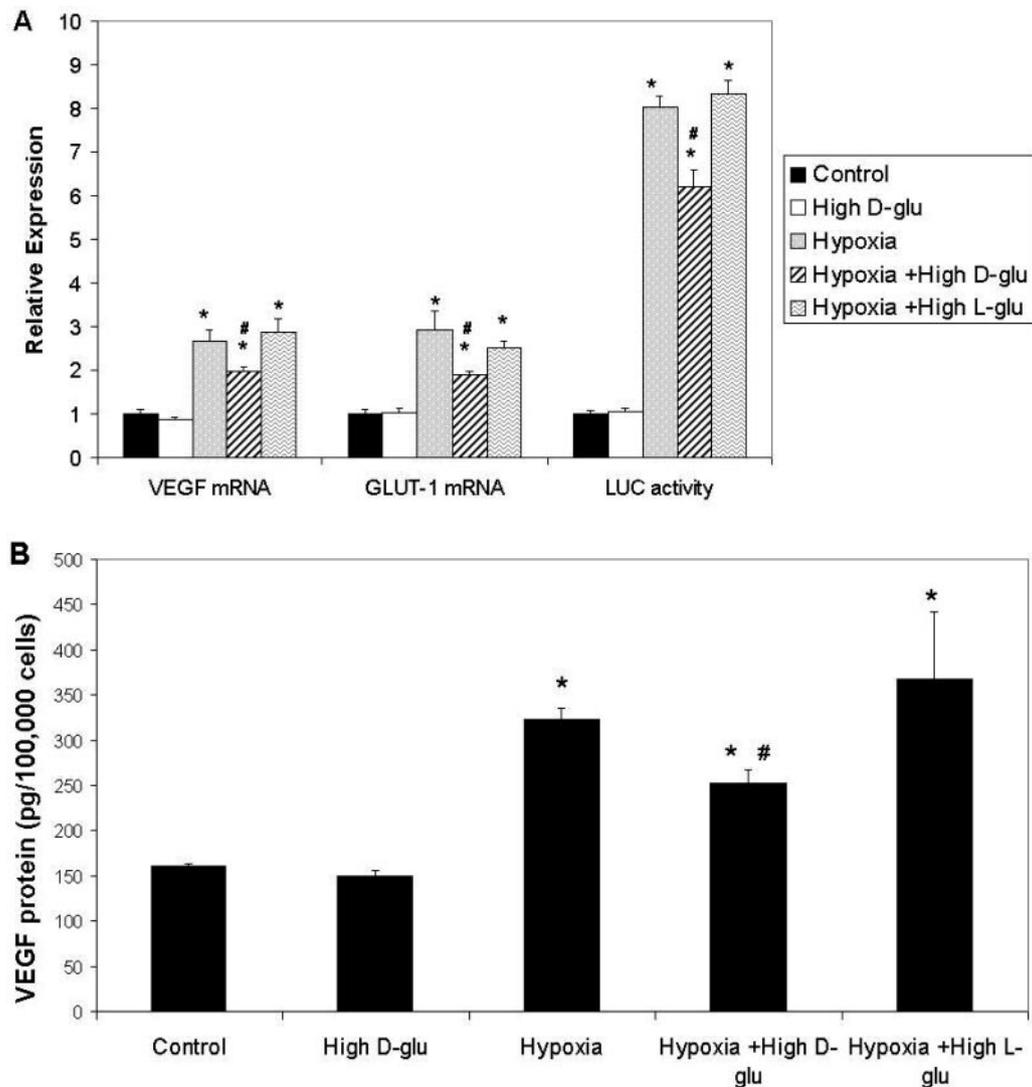
Figure 1. High glucose blunted the cellular response to hypoxia. (A) Quantitative real-time reverse transcription–PCR analysis of vascular endothelial growth factor (VEGF) and glucose transporter-1 (GLUT-1) gene expression and bioluminescent assays of luciferase activity. The relative amount of VEGF and GLUT-1 mRNA was normalized by β-actin and expressed as an arbitrary unit in which the control group value equaled 1. The relative amount of luciferase activity was normalized by total protein and expressed as an arbitrary unit in which the control group value equaled 1. (B) VEGF protein levels in cell culture supernatant. Concentrations of VEGF protein were measure by ELISA, normalized by number of cells, and expressed as picograms per 10^5 cells. Control, normoxia and normal glucose; High D-glu, normoxia and high D-glucose (25 mM); Hypoxia, hypoxia and normal glucose; Hypoxia+High D-glu, hypoxia and high D-glucose; Hypoxia+High L-glu, hypoxia and high L-glucose. *$P < 0.05$ *versus* normoxia and normal glucose group; #$P < 0.05$ *versus* hypoxia and normal glucose group.

pression under hypoxia was significantly increased (2.65 $\pm$ 0.27-fold *versus* control group). This hypoxic response of VEGF mRNA expression was significantly blunted by high D-glucose (1.98 $\pm$ 0.11 in high D-glucose under hypoxia *versus* 2.65 $\pm$ 0.27 in normal glucose under hypoxia; $P < 0.05$). There was no significant change in the hypoxia-induced VEGF mRNA expression in IRPTC that were exposed to high L-glucose compared with normal glucose (2.86 $\pm$ 0.31 *versus* 2.65 $\pm$ 0.27). Under normoxia, high D-glucose did not affect the VEGF mRNA expression in IRPTC (0.85 $\pm$ 0.08 *versus* 1.00 $\pm$ 0.10).

To define expression at the protein level, we next performed ELISA of VEGF in cell culture supernatants (Figure 1B). Hypoxia

increased the level of VEGF protein (from 161.5 $\pm$ 1.7 to 324.0 $\pm$ 11.5 pg/10^5 cells; $P < 0.05$). This hypoxia-induced VEGF protein expression also was significantly blunted by high D-glucose (252.8 $\pm$ 14.7 pg/10^5 cells in high D-glucose under hypoxia *versus* 324.0 $\pm$ 11.5 pg/10^5 cells in normal glucose under hypoxia; $P < 0.05$). IRPTC that were cultured in high L-glucose under hypoxia showed the similar level of VEGF protein to IRPTC that were cultured in normal glucose under hypoxia (367.5 $\pm$ 73.6 and 324.0 $\pm$ 11.5 pg/10^5 cells, respectively). Under normoxia, high D-glucose did not alter the VEGF protein expression (150.1 $\pm$ 6.0 pg/10^5 cells in the high D-glucose group *versus* 161.5 $\pm$ 1.7 pg/10^5 cells in the control group).

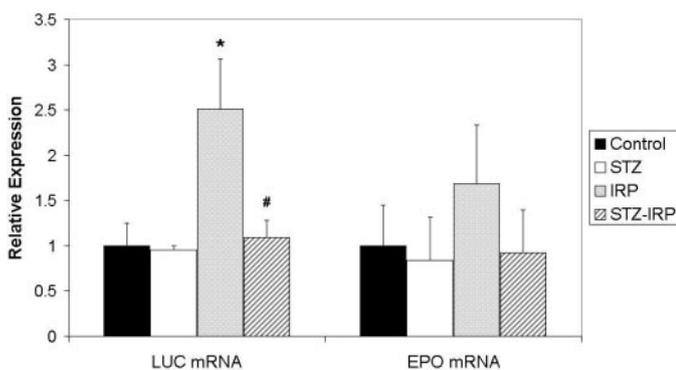### High Glucose Blunted the Hypoxia-Induced GLUT-1 Expression

GLUT-1 mRNA expression profiles were similar to that of VEGF expression (Figure 1A). GLUT-1 mRNA expression was significantly increased by hypoxia. These hypoxic responses of GLUT-1 mRNA expression also were blunted in the presence of high D-glucose (1.88 ± 0.11 versus 2.91 ± 0.44; $P < 0.05$). There were no significant changes in the hypoxia-induced GLUT-1 mRNA expression in IRPTC that were exposed to high L-glucose compared with normal glucose.

High Glucose Blunted the HIF-HRE Pathway Activation under Hypoxia

LUC activity in HRE-LUC IRPTC served as a reporter of HIF-HRE pathway activation and also showed a similar pattern to that of VEGF expression (Figure 1A). LUC activity was significantly increased under hypoxia (8.02 ± 0.25-fold versus control group). High D-glucose significantly reduced the LUC activity under hypoxia (6.20 ± 0.38 versus 8.02 ± 0.25; $P < 0.05$), whereas high L-glucose did not change the LUC activity (8.31 ± 0.33 versus 8.02 ± 0.25).

Hyperglycemia Reduced the HIF-HRE Pathway Activation in Ischemic Kidney

The effect of high glucose on the HIF-HRE pathway was demonstrated in vivo using the STZ-induced diabetes and ischemia-reperfusion model in HRE-LUC transgenic rats (Figure 2). The LUC transgene expression was significantly increased after the ischemia-reperfusion in control rats (2.51 ± 0.55-fold versus the nonischemic control). The LUC transgene expression after ischemia-reperfusion was significantly blunted in the STZ-induced hyperglycemic rats (1.09 ± 0.19 versus 2.51 ± 0.55; $P <$
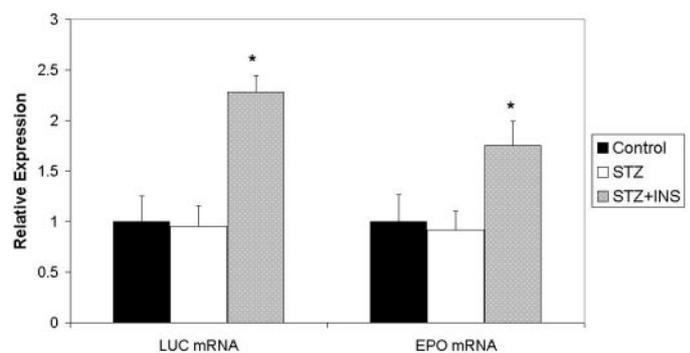
0.05). Because we could not detect significant changes of VEGF or GLUT-1 mRNA at 15 min after the ischemic procedure in control rats (data not shown), we measured expression levels of EPO mRNA as another representative gene controlled by the HIF-HRE pathway. EPO expression was increased after ischemia-reperfusion in control rats (not statistically significant) but not in STZ-induced hyperglycemic rats (Figure 2).

Insulin Treatment Restored Hypoxic Responses in STZ-Induced Diabetes

LUC transgene expression in the nonischemic kidney of control rats and STZ-induced diabetic rats was comparable (Figure 2). We hypothesized that hyperglycemia might have masked the hypoxic response in diabetic kidneys; therefore, we explored the effect of normalization of blood glucose by insulin treatment on expression of LUC transgene expression (Figure 3). LUC transgene expression in the STZ-induced diabetic rats was significantly increased after insulin treatment (2.28 ± 0.16 versus 0.95 ± 0.25; $P < 0.05$). EPO mRNA expression also was significantly increased after normalization of blood glucose (1.75 ± 0.24 versus 0.92 ± 0.19; $P < 0.05$).

RCO Increased by High D-Glucose

We next sought a mechanism for the inhibition of HRE-regulated genes by high D-glucose under hypoxic conditions. Theoretically, a high concentration of glucose may produce reactive carbonyl compounds in the culture media during incubation, which can have an effect on gene expression of cultured cells. GO and MGO were not detectable by our assays in the cell culture supernatant in all groups. The 3-DG was not increased by hypoxia (1.78 ± 0.02 in the hypoxia group versus 1.79 ± 0.04 nmol/ml in the control group), whereas it was significantly increased by high glucose with or without hypoxia (2.94 ± 0.10 with hypoxia and 3.02 ± 0.09 without hypoxia; $P < 0.05$ both; Figure 4).

Figure 2. Hyperglycemia reduced the hypoxia-inducible factor (HIF)/hypoxia-responsible element pathway activation in ischemic kidney. The relative amount of the luciferase transgene and erythropoietin (EPO) mRNA was normalized by β-actin and expressed as an arbitrary unit in which the control group value equaled 1. Control, control rats without ischemia-reperfusion injury; STZ, STZ-induced hyperglycemic rats without ischemia-reperfusion injury; IRP, control rats with ischemia-reperfusion injury; STZ-IRP, STZ-induced hyperglycemic rats with ischemia-reperfusion injury; LUC, luciferase. *$P < 0.05$ versus control rats without ischemia reperfusion injury; #$P < 0.05$ versus control rats with ischemia-reperfusion injury.

Figure 3. Insulin treatment restored hypoxic responses in STZ-induced diabetes. The relative amount of luciferase transgene and EPO mRNA was normalized by β-actin and expressed as an arbitrary unit in which the hypoxia group value equaled 1. Control, control rats; STZ, STZ-induced hyperglycemic rats; STZ+INS, STZ-induced hyperglycemic rats with insulin treatment. *$P < 0.05$ versus STZ-induced diabetic rats.
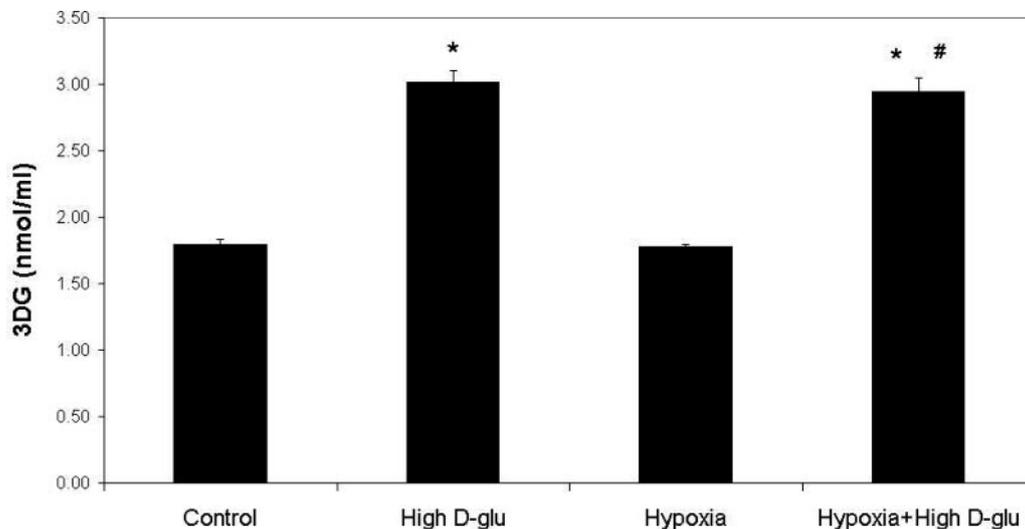
Figure 4. Concentration of 3-deoxyglucosone in cell culture supernatant. Control, normoxia and normal glucose; High D-glu, normoxia and high D-glucose (25 mM); Hypoxia, hypoxia and normal glucose; Hypoxia+High D-glu, hypoxia and high D-glucose. *$P < 0.05$ *versus* normoxia and normal glucose group; #$P < 0.05$ *versus* hypoxia and normal glucose group.

Oxidants Could Blunt Hypoxic Responses Similar to High D-Glucose

Diabetes is known to be a state of high oxidative stress, and we hypothesized that the effect of high glucose on hypoxia-induced HIF-HRE pathway activation and subsequent VEGF and GLUT-1 gene expression would be mediated by the oxidative stress. To clarify this hypothesis, we investigated the direct effect of hydrogen peroxide, a potent oxidant, on these hypoxic responses (Figure 5). Lactate dehydrogenase assays and trypan blue staining confirmed that treatment of the cells with hydrogen peroxide at this concentration did not change cell viability (data not shown). Hydrogen peroxide significantly reduced the LUC activity in HRE-LUC IRPTC that were cultured under

hypoxia (0.58 ± 0.05-fold *versus* hypoxia). VEGF and GLUT-1 gene expressions in IRPTC under hypoxia also were reduced by hydrogen peroxide (0.43 ± 0.09-fold *versus* hypoxia alone and 0.38 ± 0.08-fold *versus* hypoxia alone, respectively; $P < 0.05$ both).

Antioxidants Could Restore Hypoxic Responses Blunted by High D-Glucose

To confirm the hypothesis that high glucose reduced hypoxic responses by the oxidative stress pathway further, we used an antioxidant treatment (Figure 6). Antioxidant α-tocopherol effectively ameliorated the reduction of LUC activity in HRE-LUC IRPTC that were cultured under hypoxia and high
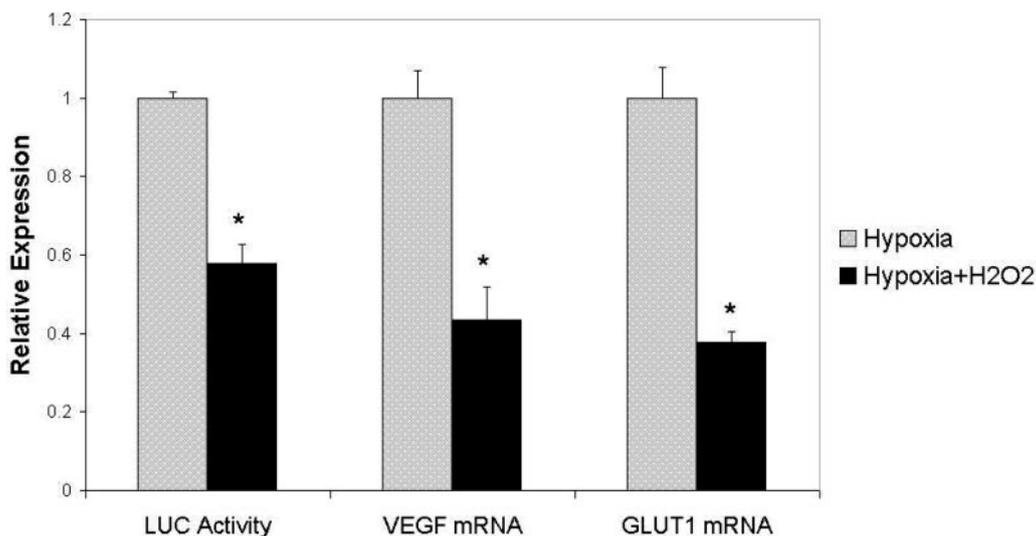

Figure 5. Hydrogen peroxide blunted the hypoxic responses. The relative amount of luciferase activity was normalized by total protein and expressed as an arbitrary unit in which the hypoxia group value equaled 1. The relative amount of VEGF and GLUT-1 mRNA was normalized by β-actin and expressed as an arbitrary unit in which the hypoxia group value equal 1. Hypoxia, hypoxia and normal glucose; Hypoxia+H_2O_2, hypoxia and 100 μM hydrogen peroxide. *$P < 0.05$ *versus* hypoxia group.
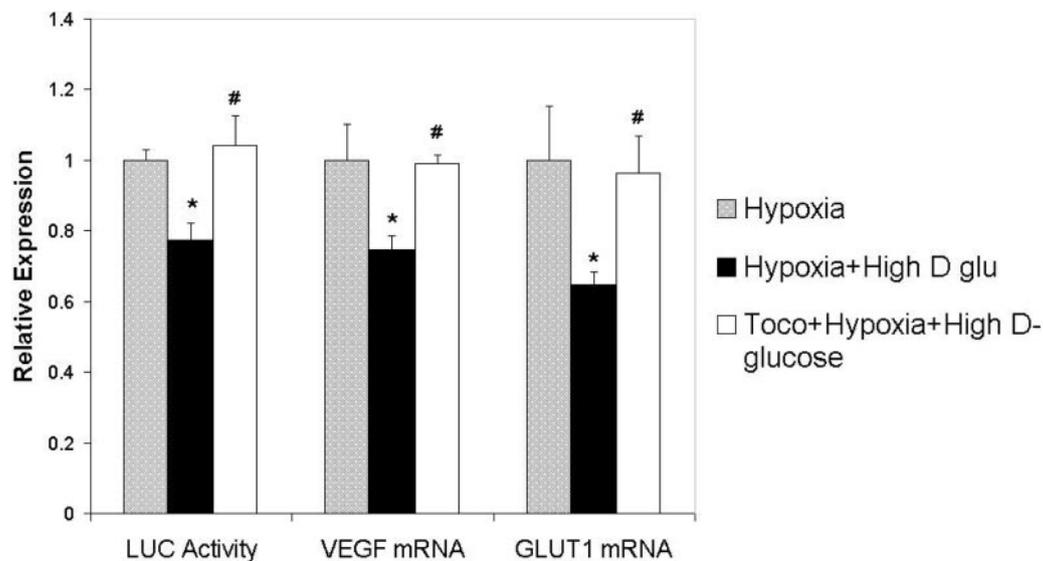
Figure 6. α-Tocopherol restored the blunted responses to hypoxia. The relative amount of luciferase activity was normalized by total protein and expressed as an arbitrary unit in which the hypoxia group value equaled 1. The relative amount of VEGF and GLUT-1 mRNA was normalized by β-actin and expressed as an arbitrary unit in which the hypoxia group value equaled 1. Toco+Hypoxia+High D-glu, hypoxia and high D-glucose and 50 μM α-tocopherol. *P < 0.05 *versus* hypoxia group; #P < 0.05 *versus* hypoxia and high D-glucose group.

D-glucose (1.04 ± 0.09 *versus* 0.77 ± 0.05; P < 0.05). The blunted VEGF and GLUT mRNA response to hypoxia by high D-glucose also was restored by α-tocopherol (0.99 ± 0.02 *versus* 0.75 ± 0.04 and 0.96 ± 0.11 *versus* 0.65 ± 0.04, respectively; P < 0.05 both).

Discussion

Our studies indicate that high glucose blunts the hypoxia-induced VEGF response in cultured proximal tubular cells, at both mRNA and protein levels. Glucose concentrations that were used in this study were physiologic, corresponding to 100 and 450 mg/dl. The blunting of the hypoxic response by high glucose also was evident by a decrease in GLUT-1 gene expression, another hypoxia-inducible gene that is expressed in cultured proximal tubular cells, as well as changes in the levels of HIF-HRE pathway activation as measured by LUC activity in IRPTC that were transfected with HRE-LUC. This blunting effect also was demonstrated *in vivo* using the ischemia-reperfusion model and STZ-induced hyperglycemia in HRE-LUC transgenic rats.

VEGF is a widely known endothelial cell angiogenic, survival, and trophic factor. In the kidney, VEGF expression in renal tubular epithelium seems likely to play a substantial role in the maintenance of peritubular capillaries (7,17). The hypoxia-induced increase in VEGF levels in proximal tubules may act as a feedback loop that reacts to cellular oxygenation and maintains peritubular capillary flow, ensuring adequate perfusion and oxygenation. If this feedback loop is altered, then the integrity of the peritubular vasculature likely becomes impaired, leading to loss of functional peritubular capillaries and causing chronic hypoxia. A high-glucose environment, which reduced the hypoxia-induced VEGF response in our study,

therefore is likely to lead to loss of peritubular capillaries, tubulointerstitial hypoxia, and, eventually, progression of CKD in patients with diabetes. This hypothesis is compatible with the previously proposed hypothesis that the reduction of VEGF expression is possibly a common cause of CKD progression (8,18). Nevertheless, further studies to clarify these mechanisms are warranted.

Indeed, VEGF has been regarded as a potential mediator of diabetic nephropathy (DN) (19). Several previous studies demonstrated increased VEGF expression in renal mesangial cells and renal tubular cells that were exposed to a high-glucose environment under normoxic conditions (7,11,13). However, this effect of high glucose is likely to be transient (11). In contrast, we observed no significant change in VEGF expression in IRPTC after 24 h of exposure to 25 mM D-glucose under normoxic conditions. This discrepancy may be due to difference of the cell lines and culture conditions. Even if high glucose could induce VEGF expression under certain conditions of normoxia by some mechanisms, it does not preclude the possibility that high glucose reduces stimulation of VEGF expression by hypoxia by other mechanisms. In our study, although IRPTC that were exposed to high glucose responded to hypoxia with increased VEGF expression, the response was blunted and likely insufficient to modulate the effects of hypoxia.

In experimental models of diabetes, increased renal VEGF expression was documented (20,21). Recent studies in patients with DN revealed increased renal VEGF expression and urinary VEGF excretion (13,22). The increased renal VEGF expression in the experimental diabetic models and in patients with diabetes that is evident in these studies may reflect the renal response to the uncorrected hypoxia. Studies that used sophis-

ticated techniques such as blood oxygen level–dependent (BOLD) magnetic resonance imaging revealed hypoxia in diabetic kidneys (23,24). The increased renal VEGF expression might contribute to pathologic intrarenal alterations in diabetic kidneys such as increased kidney weight, increased GFR, glomerular hypertrophy, and increased urinary albumin excretion. However, inhibition of VEGF in DN failed to delay the progression of renal failure (25,26). We speculate that, long term, blunted VEGF responses as well as those of other HIF-regulated renoprotective genes may render the kidney more susceptible to hypoxic insults and lead to progression of renal failure.

Although we did not study the effect of high glucose on hypoxia-induced VEGF response in other cell types, it is reasonable to speculate that the blunting of the hypoxic VEGF response as a result of high glucose would operate in other cell types as well. Because VEGF stimulates angiogenesis, the increase in VEGF that is seen in relatively hypoxic areas of the body would lead to new vessel formation. This may help to explain the clinical observation that diabetic retinopathy may worsen soon after intensive glycemic control, which would increase local VEGF levels in the eye (27,28). Deliberate restoration of poor glycemic control has been reported to improve this early worsening of diabetic retinopathy (29).

In this study, insulin treatment to normalize blood glucose ameliorated suppression of the HIF-HRE pathway activation in STZ-induced diabetic transgenic rats, as demonstrated by upregulation of LUC transgene as well as the HIF-dependent genes such as EPO after insulin treatment. These findings confirmed that diabetic kidneys suffered from suppression of the HIF-HRE pathway and that glycemic control could improve these hypoxic responses. The reason for lack of upregulation of VEGF and GLUT-1 after ischemia in the kidney is unclear. This may be explained by potential contributions of a variety of regulatory factors such as inflammatory cytokines, which might negate effects of the HIF-HRE pathway on expression of these molecules in this setting. Our finding also may emphasize the high sensitivity and specificity of our reporter gene system in the HRE-LUC transgenic rats.

GLUT-1 and EPO genes are among the hypoxia-inducible genes that are expressed in the kidney. These genes also fill a protective role in cells that are exposed to hypoxia. Inducing expression of VEGF, GLUT-1, and EPO genes has been shown to ameliorate renal ischemic injury (5). Our finding that high glucose also blunted hypoxia-induced GLUT-1 expression as well as VEGF expression may provide some pathophysiologic insights. Hyperglycemia has been associated with poor outcome after the ischemia-reperfusion injury of various organs, including kidneys (30–32). These deteriorating effects of hyperglycemia possibly are mediated, at least in part, by reducing the expression of protective genes in the cells that face the ischemic insults.

That high glucose altered the hypoxia-induced response of many hypoxia-responsive genes raises the interesting question of whether this effect is mediated by the HIF-HRE pathway. Our data of the HRE-LUC reporter vector showed that high glucose also reduced the HIF-HRE pathway activation under

hypoxic conditions, both *in vitro* and *in vivo*. This result suggested that the effect of high glucose on VEGF expression is mediated, at least in part, by alteration of the HIF-HRE pathway. In recent reports, high glucose also has been shown to reduce the hypoxia-induced HIF-1 accumulation in human fibroblast and endothelial cell lines, possibly by impairing the hypoxia-dependent protection of HIF from proteasomal degradation (33).

The RCO 3-DG was increased in IRPTC that were cultured in high-glucose conditions, whether normoxic or hypoxic. However, the presence of hypoxia did not affect the amounts of 3-DG. Furthermore, the RCO are reported to stimulate VEGF expression (34). Therefore, this increased reactive carbonyl compound is unlikely to explain the observed blunted VEGF response to hypoxia under high glucose.

A crucial role for oxidative stress in hypoxic conditions is suggested by our study. Accumulating evidence demonstrated that overproduction of superoxide by the mitochondrial electron-transport chain seems to be a primary and key event in the activation of all other pathways that are involved in the pathogenesis of diabetic complications (35,36). Studies that used animals with STZ-induced diabetes revealed that oxidative stress occurs in kidneys of diabetic rats, related to augmented oxygen consumption and impaired oxygen tension in the tissue (37). A potential mechanism of aggravation of tubular hypoxia by oxidative stress involves dissociation of mitochondrial respiration (38,39).

We extended these findings and hypothesize that the effect of high glucose on hypoxia-induced HIF-HRE pathway activation and VEGF response also would be mediated by the oxidative stress. We used oxidants, hydrogen peroxide, and antioxidant α-tocopherol to explore this possible mechanism. Our results demonstrated that hydrogen peroxide could directly reduce the HIF-HRE pathway activation as well as the subsequent VEGF and GLUT-1 gene expression under hypoxia. Our data also indicate that the blunting effect of high glucose on these hypoxia-induced responses was ameliorated by antioxidants. Therefore, we conclude that this effect of high glucose is likely to be mediated, at least in part, by regulation of the HIF-HRE pathway *via* oxidative stress. This finding supports the previously published hypothesis that redox signaling may exert some regulatory effects on the HIF-HRE pathway (40).

Reactive oxygen species were proposed to serve as mediators in the oxygen-sensing mechanism of the HIF-HRE pathway, based on several lines of evidence (40). However, it now is apparent that molecular oxygen (O_2) directly participates in an oxygen-sensing mechanism of the HIF-HRE pathway by HIF-1α hydroxylation, which promotes proteolysis of HIF and inactivation of the HIF-HRE pathway (41). Although oxidative stress may not participate in the oxygen-sensing mechanism, the available data that oxidative stress could alter the HIF-HRE pathway are important. To support this hypothesis, intracellular hydroxyl radical from Fenton reaction was shown recently to regulate the HIF-HRE pathway (42).

Although it is widely recognized that inhibition of the HIF-HRE pathway renders cells susceptible against hypoxia, we cannot exclude a possibility that the oxidative stress–induced

inhibition of the HIF-HRE pathway may serve as a protective mechanism. Stimulation of the HIF-HRE pathway not only prepares the cell for hypoxic environments but also reduces or corrects hypoxia by increasing local blood flow and oxygen delivery. An inhibitory effect of oxidative stress on the HIF-HRE pathway may help to protect the cell from oxidative injury that may present in some pathologic states even in relatively low oxygen tension, such as a high-glucose environment, by shutting out oxygen delivery to the local site at some expense of cellular hypoxia. Simple correction of hypoxia in these circumstances may induce more oxidative injuries. A preferred therapeutic approach would be to reduce oxidative stress or other pathophysiologic processes that lead to oxidative stress.

The regulatory effects of oxidative stress on the HIF-HRE pathway and expression of VEGF have potentially important implications. It also may explain the reduction of VEGF expression and loss of peritubular capillaries in aging kidneys (43), because aging is known to be associated with increased oxidative stress (44). Oxidative stress would be one of the possible mechanisms that mediate the reduction of VEGF expression, loss of peritubular capillaries, and eventually progression of CKD, because increased oxidative stress is a common finding in uremic patients (45). Finally, these observations suggest a role for antioxidants in the treatment of DN and possibly many other diseases, such as CKD and ischemia-reperfusion injury.

Conclusion

We provide evidence that high glucose blunts the hypoxia-induced VEGF response in IRPTC. Similar effects also are demonstrated on GLUT-1 gene expression. These effects are likely to be mediated by oxidative stress–regulated HIF-HRE pathway and could be ameliorated by the antioxidants. These findings provide pathophysiologic insights into the role of VEGF and oxidative stress in DN.

Acknowledgments

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