Hyperglycemia alters PI3k and Akt signaling and leads to endothelial cell proliferative dysfunction

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Hyperglycemia alters PI3k and Akt signaling and leads to endothelial cell proliferative dysfunction. Am J Physiol Heart Circ Physiol 289: H1744–H1751, 2005. First published June 17, 2005; doi:10.1152/ajpheart.01088.2004.—Diabetes mellitus is a major risk factor for the development of vascular complications. We hypothesized that hyperglycemia decreases endothelial cell (EC) proliferation and survival via phosphatidylinositol 3-kinase (PI3k) and Akt signaling pathways. We cultured human umbilical vein ECs (HUVEC) in 5, 20, or 40 mM d-glucose. Cells grown in 5, 20, and 40 mM mannitol served as a control for osmotic effects. We measured EC proliferation for up to 15 days. We assessed apoptosis by annexin V and propidium iodide staining and flow cytometry, analyzed cell lysates obtained on culture day 8 for total and phosphorylated PI3k and Akt by Western blot analysis, and measured Akt kinase activity using a GSK fusion protein. HUVEC proliferation was also tested in the presence of pharmacological inhibitors of PI3k-Akt (wortmannin and LY294002) and after transfection with a constitutively active Akt mutant protected ECs by enhancing cell survival may be secondary to high glucose concentrations.

Endothelial cell (EC) injury and proliferative dysfunction are considered to be the initial events in the development of atherosclerosis (2), postangioplasty restenosis (23, 25), plaque erosion, and thromboembolism (2), which are contributors to macrovascular complications. However, the mechanisms by which diabetes effects EC dysfunction remain poorly identified. The central importance of hyperglycemia in this process is becoming increasingly evident (8a, 43a). Population studies show that higher levels of blood glucose are associated with an incremental risk of cardiovascular disease (4). Because poor glycemic control is associated with increased vascular complications, it is possible that deficient EC proliferation and survival may be secondary to high glucose concentrations.

Materials and Methods

ECs and chemicals. Human umbilical vein ECs (HUVEC) were obtained from Clonetics (Cambrex, Walkersville, MD) and cultured in endothelial growth medium (EGM, Clonetics), containing fetal bovine serum, bovine brain extract, human epidermal growth factor, hydrocortisone, gentamicin, and amphotericin B, at 37°C in humidified 5% CO2 in air. Cells from passages 2–5 were used in this study.

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d-glucose concentration in baseline EGM was 5 mM. d-Glucose, mannitol, anti-Akt polyclonal antibody, anti-PI3k polyclonal antibody, monoclonal phospho-tyrosine antibody, and anti-rabbit and anti-mouse horseradish peroxidase (HRP) antibody were purchased from Sigma Chemicals (St. Louis, MO). Anti-phospho-specific (Ser473 and Thr308) Akt polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Annexin V-FITC and propidium iodide were obtained from Molecular Probes (Eugene, OR). Akt activity kit, including immobilized Akt Ig-1 monoclonal antibody, phospho-GSK antibody, GSK-3 fusion protein, ATP, and kinase buffer was purchased from Cell Signaling Technology (Beverly, MA). Constitutively, active and inactive Akt1 mutants in pUSEamp vector were purchased from Upstate Biotechnology.

EC proliferation assay. The effect of elevated d-glucose on HUVEC proliferation was studied by growing cells in EGM containing d-glucose concentrations of 5 (baseline control), 20, or 40 mM. The effect of hyperosmolarity was assessed by using 5, 20, and 40 mM mannitol in the growth medium. HUVEC were plated on polystyrene 24-well plates at 10^4 cells/cm^2. After 24 h, the medium was changed to 20 and 40 mM d-glucose or to 5, 20, and 40 mM mannitol. Thereafter, media was changed every 48 h. HUVEC proliferation was assessed on days 2, 5, 7, 8, 10, 14, and 15 to obtain complete cellular proliferation kinetics. Cell number was determined by manual Coulter counting of a trypsinized aliquot of cells as described previously (28). For subsequent experiments, cell numbers were counted on day 8 (midlog-phase of growth). Cell viability was assessed by using Trypan blue staining.

ECs apoptosis assay. HUVEC were grown in EGM containing 5 (baseline control), 20, or 40 mM d-glucose for 8 days. Five microliters of annexin V-FITC and ten microliters of propidium iodide (50 µg/ml in 1× PBS) were added to a trypsinized aliquot of HUVEC (10^6 cells/ml) in binding buffer (Cell Signaling). Cell necrosis and apoptosis were measured by using flow cytometry within 30 min after fluorescent labeling by using standard quantitative methods (fluorescence activated cell sorter caliber by Becton-Dickinson).

Preparation of cellular lysates and immunoblot analysis. HUVEC were cultured in media containing 5, 20, and 40 mM d-glucose or 5, 20, and 40 mM mannitol for 8 days. Cells were then washed with ice-cold PBS, and whole cell extracts were prepared by exposing the cells to modified Tris-NaCl-EDTA buffer [20 mM HEPES (pH 7.4), 1% Triton X-100, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1.5 µM pepstatin]. Protein quantification of the samples was carried out by using the bicinchoninic acid assay (Pierce, Rockford, IL). All immunoblots were standardized to the same amount of protein per well. Equal amounts of protein were applied for SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes. Western blot analysis was carried out by incubating the membranes with antibodies to PI3k, Akt, phosphospecific (Ser473 and Thr308) Akt, and myc. The signal was visualized by using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence assay (Amersham; Piscataway, NJ). Band intensities were determined by using an IS-1000 imaging system (Alpha Innotech; San Leandro, CA).

Immunoprecipitation. HUVEC were cultured in media containing 5, 20, and 40 mM d-glucose for 8 days. Cells were then washed with ice-cold PBS, and whole cell extracts were prepared (see Preparation of cellular lysates and immunoblot analysis). The samples were diluted to a final concentration of 500 µg/ml and precleared by incubating with 20 µl of protein A-Sepharose bead slurry for 30 min at 4°C. The samples were then incubated with 0.5 µl of polyclonal PI3k antibody. The immunocomplex was captured with protein A-Sepharose beads slurry and resuspended by boiling in 2× sample buffer. SDS-PAGE was performed with the supernatant and transferred to the PVDF membranes. The membranes were probed for phospho-tyrosine using monoclonal phospho-tyrosine antibody.

Akt kinase activity assay. HUVEC were cultured in media containing 5, 20, and 40 mM d-glucose for 8 days. Cells were then washed with ice-cold PBS, and whole cell extracts were prepared (see Preparation of cellular lysates and immunoblot analysis). Akt was selectively immunoprecipitated from HUVEC lysates using resuspended immobilized Akt antibody slurry. The resulting immunoprecipitate was incubated with GSK-3 fusion protein in the presence of ATP and kinase buffer. This allowed Akt to phosphorylate GSK-3. Phosphorylation of GSK-3 was measured by Western blot analysis using a phospho-GSK-3α/β (Ser 21/9) antibody.

Pharmacological inhibition of PI3k-Akt. In separate experiments, HUVEC were grown in 5 mM d-glucose with increasing doses of wortmannin (1, 10, and 100 nM) or LY294002 (0.1, 1, 10, and 100 µM). Cells were counted after 8 days to determine the effect of pharmacological inhibition of PI3k-Akt on EC proliferation.

Akt transfection. HUVEC were transiently transfected with myc-tagged constitutively active (myr-Akt1) and dominant negative [Akt1 (K179M)] mutants of Akt according to the manufacturer’s protocol. Successful transfection was tested with immunoblotting with anti-myc and anti-Akt antibodies. Transfected cells were allowed to grow in media containing 5, 20, and 40 mM d-glucose, and cells were counted on day 8 to determine whether transfection with constitutively active Akt could protect ECs from the effects of high glucose concentrations.

Statistical analysis. Proliferation data are means ± SD. Immunoblot data are means ± SD of band intensity relative to control. All experiments were repeated for n = 6. Statistical analysis was performed using GraphPad Prism 3.0 (San Diego, CA). Groups were analyzed for differences by one-way ANOVA followed by Tukey’s test. Significance was accepted at P < 0.05.

RESULTS

High glucose concentrations and EC proliferation and survival. Baseline HUVEC proliferation in EGM containing 5 mM d-glucose followed standard growth kinetics with a clearly defined lag (0–6 days), log (7–10 days), and plateau (11–15 days) phase (Fig. 1A). Because day 8 was well within the log phase of cell proliferation, further comparisons were made on that day. The number of proliferating HUVEC on day 8 were significantly lower when cultured in 20 and 40 mM d-glucose than when cultured in 5 mM d-glucose (P < 0.01 each; Fig. 1A). HUVEC grown in EGM containing equimolar concentrations of mannitol (3, 20, and 40 mM) did not demonstrate a decrease in proliferation (Fig. 1B). Therefore, the effect of d-glucose on proliferation was not secondary to osmotic load. To ascertain whether the decreased cell counts in 20 and 40 mM d-glucose were due to reduced proliferation or increased cell death, we performed flow cytometry with propidium iodide staining. There was no significant difference in the percentage of necrotic cells when cultured in 20 or 40 mM d-glucose than when cultured in 5 mM d-glucose (P > 0.05 for both, Fig. 1C). These results indicate that HUVEC necrosis was not enhanced by the increasing d-glucose concentrations. Because apoptosis may occur in the absence of necrosis, we performed flow cytometry using annexin V-FITC staining to detect apoptosis (Fig. 1C). Cells cultured in 5 mM d-glucose had significantly less apoptotic cells than necrotic cells (P < 0.05). Cells cultured in 20 mM d-glucose demonstrated an increasing trend (P > 0.05), but those grown in 40 mM d-glucose presented a significantly higher percentage of apoptotic cells than those cultured in 5 mM d-glucose (2.35% apoptotic cells in 5 mM vs. 4.81% in 40 mM; P < 0.01).
High glucose concentrations and PI3k expression in ECs. Total PI3k was not significantly altered in HUVEC cultured in 20 or 40 mM d-glucose compared with 5 mM d-glucose (Fig. 2). To determine whether this difference was because of a reduction in the total immunoprecipitated PI3k or a true decrease in phosphorylated PI3k, we stripped these membranes and reprobed them for total PI3k. Total PI3k was not different between groups (Fig. 2).

High glucose concentrations and Akt expression in ECs. Total Akt in HUVEC lysates remained unchanged when cultured in 5, 20, or 40 mM d-glucose and in 5, 20, or 40 mM mannitol (Fig. 3A). Akt is primarily activated by PI3k-mediated phosphorylation of its Thr<sup>308</sup> and Ser<sup>473</sup> residues. These phosphorylations additively activate Akt serine/threonine kinase activity. We therefore measured the levels of phosphorylated Akt at Thr<sup>308</sup> and Ser<sup>473</sup> by immunoblotting with their respective phospho-antibodies. Whereas the level of phospho-Ser<sup>473</sup> Akt was not significantly altered among groups (Fig. 3B), there was a significant decrease in phospho-Thr<sup>308</sup> Akt in HUVEC cultured in 20 and 40 mM d-glucose compared with control (5 mM; *P < 0.05 and #P < 0.003, respectively, Fig. 3C). Exposure to 5, 20, or 40 mM mannitol did not alter the expression of phospho-Ser<sup>473</sup> Akt or phospho-Thr<sup>308</sup> Akt relative to 5 mM d-glucose control (Fig. 3, B and C).

High glucose concentrations and Akt activity in ECs. We performed an Akt activity assay to determine whether the quantitative differences in phosphorylated PI3k and phospho-Thr<sup>308</sup> Akt affected the activity of Akt in HUVEC grown in increasing concentrations of d-glucose. HUVEC were cultured in 5, 20, and 40 mM d-glucose, and Akt was selectively

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**Fig. 1.** Increasing d-glucose concentrations decrease endothelial cell (EC) proliferation independent of osmotic changes or cell death. A: human umbilical vein ECs (HUVEC) were seeded at 1,000 cells/well and grown in endothelial growth medium containing baseline control (5 mM) and high (20 and 40 mM) concentrations of d-glucose. Cell counts were performed through 15 days. Log phase of growth was between days 7 and 10. Number of proliferating HUVEC on day 8 were significantly lower when cultured in 20 (P < 0.01) and 40 mM (P < 0.01) d-glucose compared with control (5 mM d-glucose). B: mannitol at 5, 20, and 40 mM did not change HUVEC proliferation. C: percentage of cells undergoing necrosis was not significantly different among groups. Relative percentage of apoptotic cells was lower than necrotic cells in 5 mM d-glucose (*P < 0.05). There was an increase in percentage of apoptotic cells in 40 mM d-glucose vs. 5 mM d-glucose (#P < 0.01).

**Fig. 2.** Concentration of 40 mM d-glucose decreases phospho-phosphatidylinositol 3-kinase (PI3k) expression in ECs. Immunoblots were performed for tyrosine-phosphorylated proteins after immunoprecipitation of PI3k from lysates obtained from HUVEC cultured in 5, 20, and 40 mM d-glucose. Total PI3k was evaluated by reprobing membranes. Bar graph shows the mean relative band intensity ± SE of tyrosine-phosphorylated PI3k in lysates. *P < 0.05 compared with control (5 mM d-glucose).
immunoprecipitated from HUVEC lysates and incubated with GSK-3 fusion protein in the presence of ATP and kinase buffer. Akt kinase activity was assessed by measuring the phosphorylation of GSK-3 by Western blot analysis using a phospho-GSK-3α/β (Ser 21/9) antibody. We found that Akt activity was markedly reduced in lysates from HUVEC cultured in 20 mM D-glucose ($P < 0.05$) and 40 mM D-glucose ($P < 0.001$; Fig. 4).

**Pharmacological inhibition of PI3k-Akt and EC proliferation.** We tested EC proliferation in various concentrations of pharmacological inhibitors selective for PI3k-Akt to determine whether the decrease in proliferation seen in ECs grown in high glucose concentrations could be reproduced. HUVEC were grown in normal glucose concentrations (5 mM) with increasing concentrations of wortmannin (1, 10, and 100 nM) or LY294002 (0.1, 1, 10, and 100 μM). EC proliferation was attenuated in the presence of all three concentrations of wortmannin ($P < 0.05$ for each dose vs. 5 mM D-glucose; Fig. 5A). Additionally, there was a dose-dependent reduction in EC proliferation when a more specific PI3k-Akt blocker LY294002 was used ($P < 0.05$ for 1 μM, and $P < 0.001$ for 10 μM vs. 5 mM D-glucose; Fig. 5B).

**High glucose concentrations and Akt transfection.** We performed transient transfections of Akt mutants to test whether overexpression of Akt could protect ECs from the proliferation inhibition induced by exposure to high glucose concentrations. HUVEC were transiently transfected with myc-tagged constitutively active (myr-Akt1) and dominant negative [Akt1 (K179M)] mutants of Akt, and cells were counted on day 8. Transfection with an empty pUSEamp vector served as a control. Successful transfection was confirmed by immunoblotting with anti-myc and anti-Akt antibodies (Fig. 6A). Notably, endogenous Akt was not depleted in the presence of dominant negative Akt. When grown in 5 mM D-glucose, HUVEC with constitutively active Akt demonstrated significantly higher proliferation compared with nontransfected ECs and ECs transfected with empty vector or Akt−/− mutant (Fig. 6B). When grown in 20 mM D-glucose, HUVEC with constitutively active Akt demonstrated significantly higher proliferation compared with nontransfected ECs and ECs transfected with empty vector or Akt−/− mutant (Fig. 6B). Notably, the protective effect allowed Akt+/+ ECs grown in 20 mM D-glucose to proliferate, as well as nontransfected ECs grown in 5 mM D-glucose (Fig. 6C). When grown in 40 mM D-glucose, HUVEC with constitutively active Akt demonstrated significantly higher proliferation compared with nontransfected ECs and ECs transfected with empty vector or Akt−/− mutant. Notably, protection was only partial, allowing Akt+/+ ECs grown in 40 mM D-glucose to proliferate more than nontransfected ECs grown in 40 mM D-glucose but not as much as nontransfected ECs grown in 5 mM D-glucose (Fig. 6D).

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Fig. 3. A: high D-glucose concentration does not alter total Akt expression in ECs. HUVEC were cultured in 5, 20, or 40 mM D-glucose and in 5, 20, and 40 mM mannitol. Bar graphs show the means relative band intensity ± SE of Akt in lysates. B: high D-glucose concentration does not alter expression of phosphorylated Akt at Serine 473 in ECs. C: high D-glucose concentration decreases expression of phosphorylated Akt at Threonine 308 in ECs. *$P < 0.05$, # $P < 0.01$. 

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DISCUSSION

PI3k and Akt are downstream effectors of insulin signaling (12), as well as important signaling molecules in the regulation of glycogen metabolism in myocytes, lipocytes, and hepatocytes (16, 42). Uncoupling of insulin signaling at PI3k-Akt in these cell types in response to high glucose concentrations has been implicated in the pathogenesis of insulin resistance and Type II diabetes (44). However, PI3k-Akt also plays an important role in ECs by regulating angiogenesis (18), proliferation (13), microvascular permeability (29), survival (10), cellular transformation, and embryonic differentiation (41). Our results are the first to show unequivocally the role of PI3k and Akt in hyperglycemia-induced effects in ECs. Our novel findings suggest a unified mechanism through which hyperglycemia causes insulin resistance in nonvascular cells and causes proliferative dysfunction in ECs. The need for research into the role of Akt signaling in the pathophysiology of complications of diabetes mellitus has been recently emphasized (46).

Diabetes is associated with a two- to fourfold increase in myocardial infarction (45) and stroke (26), a 15- to 40-fold increase in limb loss from lower extremity arterial occlusive disease (36), and an increase in restenosis rates after intravascular interventions (23, 25). Although diabetes is associated with several microvascular complications (9, 24, 35), the macrovascular complications are the major cause of mortality and account for 77% of hospitalizations in these patients (15). The pathogenesis of macrovascular complications in diabetes mellitus is not fully understood. Decreased EC proliferation may play a central role in the development of atherosclerosis, decreased collateral arteriogenesis around occlusive lesions (1, 17, 40), and decreased reendothelialization of injured blood vessels (23, 25). Tight control of hyperglycemia is the only currently effective method of limiting diabetic cardiovascular complications, thereby indicating that hyperglycemia contributes significantly to their pathogenesis. The results of our study are consistent with these clinical observations. We demonstrate that exposure to increasing concentrations of glucose results in diminished EC proliferation and survival. Our results are in agreement with previous reports that high glucose concentrations reduced proliferation in HUVEC (7), human pulmonary artery ECs (31), human dermal microvascular ECs (20), porcine aortic ECs (14), and bovine retinal ECs (27). Conversely, other investigators report that hyperglycemia enhances proliferation of retinal microvascular ECs (34, 38) and of HUVEC (39). These conflicting reports may be related to differences in species or experimental conditions (passage number, extent of growth arrest before glucose exposure, concentration and duration of glucose exposure, and whether the experiments were controlled for insulin in the media). The observation that retinal ECs have increased proliferation with high glucose is consistent with the clinical finding that diabetic patients have high rates of proliferative retinopathy (24). However, the effect of diabetes on other vascular beds (coronary and lower extremity vascular disease) may be related to deficient EC proliferation and survival.

![Fig. 4. High D-glucose concentration decreases Akt activity in ECs. Akt was immunoprecipitated from HUVEC lysates using immobilized Akt antibody slurry. Immunoprecipitate was incubated with GSK-3 fusion protein in presence of ATP and kinase buffer. Phosphorylation of GSK-3 was used as a measure of Akt activity. Bar graphs show the means relative band intensity ± SE of phospho-GSK-3α/β (Ser 21/9) in lysates. ∗P < 0.05 and #P < 0.001 compared with control (5 mM D-glucose).](https://www.ajpheart.org)

![Fig. 5. Pharmacological inhibition of PI3k-Akt inhibits EC proliferation. A: EC proliferation was attenuated by wortmannin at all concentrations (P < 0.05 vs. 5 mM D-glucose). B: LY294002 produced dose-dependent reduction in EC proliferation (P < 0.05 for 1 μM and P < 0.001 for 10 μM vs. 5 mM D-glucose).](https://www.ajpheart.org)
In our experiments, we used 5 mM D-glucose as control exposure because this is the normal concentration in EGM, and 20 mM D-glucose was used as a challenge dose, because this is equivalent to the serum glucose concentration in diabetic individuals (90 mg/dl). Both 20 and 40 mM D-glucose were used as challenge concentrations in diabetic individuals (360 and 720 mg/dl), respectively. Cells were exposed to prolonged hyperglycemia by increasing serum glucose concentration (8 days), beyond what has been usually reported in the literature. As the normal concentration in EGM is 5 mM, we used 5 mM D-glucose as control exposure because this is the normal concentration in EGM. Therefore, we conclude that hyperglycemia affects cellular proliferation more than apoptosis under these conditions. Increasing concentrations of mannitol did not alter proliferation and apoptosis (8 days), indicating that the altered proliferation and apoptosis in our experiments are not due to an osmotic challenge but more likely through an altered signaling cascade. We hypothesize that the altered signaling cascade in ECs, which is demonstrated by hyperglycemia, alters cellular signaling as well as PI3k/Akt. We are not only interested in the Akt activity because Akt overexpression protects ECs from the deleterious effect of hyperglycemia. We demonstrate that a significantly higher percentage of cells cultured in 40 mM D-glucose underwent early apoptosis, whereas cell numbers was not affected by 20 mM D-glucose. Decreased Akt activity is important to note that Akt activity is reduced at 20 and 40 mM D-glucose, supporting an important role for Thr308 phosphorylation in the mechanism by which high glucose causes a decrease in Akt kinase activity. Furthermore, Akt phosphorylation did not vary significantly among the treatment groups. These findings suggest that the decreased Akt kinase activity in HUVEC cultured in 20 and 40 mM D-glucose is due to a selective and specific change in thrreonine phosphorylation at site 308 in Akt and decreases in Akt protein. The decrease in Akt protein is the most glucose-sensitive element of the mechanism by which high glucose causes a decrease in Akt kinase activity. We interpret our data to indicate that Thr308 phosphorylation is the most glucose-sensitive element of the mechanism by which high glucose causes a decrease in Akt kinase activity.
multicenter trials have demonstrated that intensive control of hyperglycemia can reduce the incidence or progression of microvascular complications of diabetes (8a, 43a). However, there is limited data with respect to the mechanisms of the macrovascular complications of diabetes. Our study suggests that hyperglycemic conditions would cause reduced proliferation and survival in vascular ECs. It also provides novel insights into an endothelial signaling dysfunction (reduced PI3k and Akt activity) that may explain these changes in diabetic patients. These observations linking PI3k-Akt activity to hyperglycemia-induced EC-proliferation defects have great clinical relevance because restoration of insulin signaling is a major therapeutic intervention being tested in current clinical trials (19). Downstream insulin signaling through PI3k-Akt may be blocked in diabetics through hyperglycemia-induced activation of PKC-βIII. Ruboxistaurin (LY333531), an inhibitor of PKC-βIII (19), has been shown to prevent and/or reverse diabetic microvascular complications such as retinopathy (3), nephropathy (43), and neuropathy (22). If PI3k-Akt signaling defects contribute to diabetic macrovascular complications, then therapeutic restoration of activity may have clinical significance. We suggest that upregulation of PI3k and Akt signaling pathways within the endothelium should be considered as a target for future therapeutic modalities in protecting the diabetic patient from macrovascular complications.

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