Acute effect of high glucose on long-term cell growth: a role for transient glucose increase in proximal tubule cell injury

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Acute effect of high glucose on long-term cell growth: a role for transient glucose increase in proximal tubule cell injury. Am J Physiol Renal Physiol 291: F162–F175, 2006. First published February 7, 2006; doi:10.1152/ajprenal.00189.2005.—Although chronic exposure of renal cells to high glucose has been shown to cause cell injury, the effect of acute exposure has not been elucidated. In this study, we demonstrate that acute (10 min) exposure of human proximal tubule epithelial cells (hPTEC) to high glucose (25 mM) induces a time-dependent dual effect consisting of an early proliferation and a late apoptosis. Acute exposure of hPTEC to high glucose induced a twofold increase in DNA synthesis and cell number at 12 h. However, after 36 h, a significant decrease in cell growth is observed, followed by apoptosis. On glucose treatment, both p52/p42 mitogen-activated protein (MAP) kinases and the downstream signaling intermediate NF-κB were phosphorylated and translocated to the nucleus. Pretreatment of cells with MAP kinase and NF-κB-specific inhibitors abolished glucose-induced proliferation. However, these inhibitors were ineffective in preventing glucose-induced apoptosis. Interestingly, conditioned medium from cells exposed to high-glucose concentrations inhibited proliferation and concomitantly induced apoptosis in normal cells, suggesting that the inhibitory effect of glucose occurs through secretion of a secondary factor(s). In parallel to apoptosis, we observed an increased production of reactive oxygen species (ROS). Pretreatment of cells with the antioxidant N-acetyl cysteine reversed glucose-mediated ROS production and apoptosis, suggesting that ROS is involved in apoptosis. Our study demonstrates for the first time that a single high-glucose exposure for 10 min alone is sufficient to elicit proliferation and apoptosis in hPTEC and suggests that episodes of transient increase in glucose may contribute to cell damage leading to epithelial cell dysfunction.

human proximal tubule epithelial cells; diabetes; hyperglycemia

Diabetes, which is characterized by an increased concentration of glucose in the blood, is a major risk factor in the pathogenesis of renal disease (47). Hyperglycemia-induced abnormal growth and function of the glomerular and tubular cells have been proposed to be the primary determinants in the progression of diabetic nephropathy. Long-term exposure of glomerular cells to high-glucose levels was shown to upregulate the synthesis of extracellular matrix proteins such as fibronectin and collagen, and growth factors such as transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (18, 24, 30, 38). These events cause functional and structural changes in the glomerulus, resulting in glomerular hyperfiltration, thickening of glomerular basement membranes, and excessive deposition of the extracellular matrix, leading to glomerular abnormalities (14, 47, 61). Although glomerular disease is considered the major determinant of diabetic nephropathy, recent studies have shown a significant correlation between diabetes-induced renal disease and progressive pathological changes in renal proximal tubule, suggesting a role for tubular pathology in diabetic nephropathy (60). The renal tubule in diabetes is subject to both direct and indirect insults as a consequence of its position in the nephron and its resorptive function. A tubular dysfunction compromises the water and salt homeostasis. Such tubular changes have been reported to be the dominant lesion in about one-third of patients with type 2 diabetes (12).

In proximal tubule epithelial cells, glucose enters the intracellular space through apical domain of the epithelial cells by sodium glucose symporter (20, 62). The presence of microvilli in the apical plasma membrane of these cells increases the surface area as much as 25-fold and thus facilitates the increase of glucose transport activity, compared with other cell types. Prolonged exposure (1–13 days) of proximal tubule epithelial cells (PTEC) to hyperglycemic environment has been shown to inhibit cell proliferation and induce growth arrest or cellular apoptosis (13, 41, 57). These cellular effects are caused by the activation of a network of intracellular signaling pathways and include diacylglycerol-induced activation of PKC (8, 27, 41, 56), MAP kinase (13, 16, 21, 56), and NF-κB (15, 33, 36). In addition, it has been shown that long-term continuous exposure of glucose will lead renal cells to a state of oxidative stress (1, 4, 57). The oxidative stress observed in hyperglycemia appears to increase expression of various proinflammatory genes, which are NF-κB dependent, suggesting a possible role for NF-κB activation in diabetic renal injury (22). In addition, oxidative stress-mediated activation of caspase-3 has been shown to be a principle mediator of hyperglycemia-induced proximal tubular apoptosis (1). Furthermore, high-glucose concentrations induce continuous synthesis and secretion of growth factors (18, 39), raising the possibility that these factors may also be involved in the observed effects. Because growth factors are known to rapidly activate signaling pathways leading to cell growth and apoptosis, we questioned whether prolonged exposure of glucose is required to stimulate intracellular signaling pathways or whether short-term exposure would cause similar effect. To our knowledge, there are no studies demonstrating the short-term (acute) effect of high glucose on long-term cell growth and function.

The present study is designed to close the gap in our current understanding of the role of glucose in intracellular signaling and downstream cellular effects. Therefore, we sought to determine whether a short-term (10 min) exposure of human...
PTEC (hPTEC) to high glucose induces growth changes, through similar or distinct mechanisms observed with prolonged high-glucose exposure. The study was also carried out to understand the effects of high glucose on the activation of intracellular signaling network and how this regulates cellular response in hPTEC. Our study demonstrates that a 10-min exposure of high glucose to hPTEC induces a dual effect involving an early phase of cell proliferation (12 h) and a late phase of apoptosis (48 h). In these cells, glucose rapidly induces both p42/p44 MAP kinases and NF-κB and causes their nuclear translocation. Our study shows that MAP kinases are involved in glucose-induced proliferative phase. However, the apoptotic phase is mediated by a glucose-induced increase in reactive oxygen species (ROS). Moreover, glucose-induced inhibition of cell proliferation observed at 24 h and later time points appears to be due to the extracellular release of an inhibitory factor. This is the first study elucidating the acute effects of high glucose on cell signaling and the downstream response involving both proliferation and apoptosis in renal PTEC.

**MATERIALS AND METHODS**

**Materials.** Cell culture reagents were purchased from Invitrogen (Carlsbad, CA); phospho-specific ERK and anti-ERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-specific IκB-α, anti-IκB-α, phospho-specific NF-κB, and anti-NF-κB were obtained from Cell Signaling (Beverly, MA); goat anti-rabbit IgG, goat anti-mouse IgG, and donkey anti-goat IgG were from Santa Cruz Biotechnology; MAP kinase kinase 1 (MAPKK1) inhibitor PD-98059 and NF-κB activation inhibitor were purchased from Calbiochem (La Jolla, CA); electrophoresis reagents were from Bio-Rad (Richmond, CA); nitrocellulose membrane was from Amersham (Piscatway, NJ); d-glucose and all other chemicals were obtained either from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

**Cell culture.** The human proximal tubule cell line (hPTEC) obtained by SV-40 transformation was kindly provided by Dr. L. Racusen (The Johns Hopkins University School of Medicine, Baltimore, MD) (46). The hPTEC cells were maintained in a 1:1 mixture of DMEM/Ham’s F-12 nutrient mixture (DMEM/F-12) containing 5% FBS, 50 μU/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO2 under 100% humidity. For the studies, cells were grown to 75–80% confluence, and the medium was replaced with DMEM with normal glucose (5.5 mM) containing 0.5% FBS and grown for another 24 h. For chronic exposure, cells were continuously stimulated in high glucose for indicated times. However, for acute exposure, cells were stimulated in high glucose for 10 min or less and grown for indicated times in normal glucose. Simultaneously the control plates were replenished with fresh medium containing normal concentration of glucose. d-Glucose was used in all experiments unless otherwise stated.

**Western blot analysis.** Cells were stimulated with 25 mM glucose, treated with various agents for the indicated times, and washed with phosphate-buffered saline. Cells were scraped in lysis buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 15% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Protein concentration was determined using Bio-Rad protein assay kit according to the manufacturer’s instructions (Amersham).

**Preparation of nuclear extract.** Initially, cells were grown to 75–80% confluence in complete medium. Twenty-four-hour serum-starved (0.5% FBS) cells in DMEM medium (5.5 mM glucose) were stimulated with high glucose (25 mM) for indicated times. Cells were rinsed two times with PBS, scraped into PBS without Mg2+ and Ca2+ (pH 7.4), and cytosolic and nuclear extracts were prepared as described previously (44). Briefly, harvested cells were centrifuged at 650 g for 10 min at 4°C, and cell pellets were resuspended in ice-cold hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, and 0.5 mM dithiothreitol (DTT) supplemented with protease inhibitors (Complete Mini protease inhibitor mixture tablets, EDTA-
free; Roche Diagnostics). The cells were Dounce homogenized (20 strokes) and centrifuged at 1,000 g for 10 min at 4°C. The supernatant cytoplasmic extract was stored at \(-70^\circ\)C for later use, and the pellet was resuspended in high-salt buffer containing 20 mM HEPES, 25% glycerol, 400 mM NaCl, and 1 mM EDTA supplemented with protease inhibitors. The high-salt nuclear extract was dialyzed (membrane molecular weight cut-off of 8 kDa) overnight in a buffer containing 20 mM Tris \(\cdot\) HCl, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, and 50 \(\mu\)g/ml PMSF. Protein concentration was determined by the method previously indicated and extracts were stored at \(-70^\circ\)C for later use.

**Cell proliferation and viability assay.** Measurement of \(^{3}H\)thymidine incorporation, as an indicator of DNA synthesis, was performed as described previously (45). Briefly, the cells were plated in six-well plates in complete medium at a density of 5 \(\times\) 10^4 cells/well. The supernatant cytoplasmic extract was stored at \(-70^\circ\)C for later use, and the pellet was resuspended in high-salt buffer containing 20 mM HEPES, 25% glycerol, 400 mM NaCl, and 1 mM EDTA supplemented with protease inhibitors. The high-salt nuclear extract was dialyzed (membrane molecular weight cut-off of 8 kDa) overnight in a buffer containing 20 mM Tris \(\cdot\) HCl, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, and 50 \(\mu\)g/ml PMSF. Protein concentration was determined by the method previously indicated and extracts were stored at \(-70^\circ\)C for later use.

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**Detection of apoptosis by Hoechst staining assays.** Cells were grown to 60–70% confluency on cover slips and serum-starved (0.5% FBS) for 24 h. Cells were then exposed to high glucose for indicated times, washed with PBS, and fixed with 70% ethanol for 10 min at room temperature. Fixed cells were rehydrated with PBS and subsequently stained with Hoechst 33258 in PBS (10 \(\mu\)g/ml) for 10 min. After being washed two times with PBS, cells were viewed and photographed with a fluorescence microscope (Olympus BX41) using a DAPI filter and \(\times\)40 objective.

**Flow cytometry by annexin V-FITC conjugated with propidium iodide staining for apoptosis.** The assay is based on fluorescein isothiocyanate (FITC)-conjugated annexin V binding to externalized phosphatidylserine (PS) during apoptosis, a phospholipid normally restricted to the inner leaflet of the plasma membrane and detection by flow cytometry (63). Serum-starved cells were treated with normal or high glucose in the presence or absence of other agents as indicated for 48 h. Harvested cells were assayed for apoptosis using an annexin V-FITC apoptosis detection kit (Cat. no. PF032–1EA, Calbiochem) according to the manufacturer’s direction. To prepare the cell samples for flow cytometry, cells were washed with annexin-binding buffer for 10 min at room temperature. A hemocytometer was used to count live and dead cells, and the number of viable and apoptotic/dead cells per well was calculated.

**Fig. 2.** Effect of chronic and acute exposure of cells to HG on cellular apoptosis. hPTEC were grown to 80% confluency and serum-starved (in 0.5% FBS) for 24 h. A: cells were continuously exposed to NG (1 and 2) or HG (2) for 48 h or pulsed with HG for 10 min, washed, and incubated with normal-glucose medium for 48 h (4). Cells were stained with Hoechst 33258, and chromatin condensation and DNA fragmentation were visualized with a fluorescence microscope equipped with a DAPI filter using a \(\times\)40 objective. B: cells were exposed to NG or HG (acute and chronic) and harvested by trypsinization at indicated time periods. A Trypan blue exclusion assay was performed to determine cell death as described in MATERIALS AND METHODS. Data are expressed as means \(\pm\) SE of triplicate values. *P < 0.05 compared with cells exposed to NG.
and stained with annexin V FITC and propidium iodide (PI). Analysis was conducted for 30,000 cells using flow cytometer with CellQuest software (Becton-Dickinson, Rutherford, NJ) using FL1 and FL2 ranges for annexin V FITC and PI, respectively.

Flow cytometric determination of intracellular ROS. 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used for ROS capture. DCFH-DA is a cell-permeable dye cleaved intracellularly by nonspecific esterases and turns to highly fluorescent 2,7-dichlorofluorescein (DCF) on oxidation by ROS. 2,7-DCFH-DA was dissolved in ethanol and stored as 5 mM stock. DCFH-DA solution was added directly to the medium to reach 10 μM and then incubated at 37°C for 30 min. Cells were harvested at the indicated time points, washed three times with PBS, and then immediately analyzed by flow cytometer (Becton-Dickinson) with a 485-nm excitation wavelength. Each determination is based on a mean fluorescence intensity of 30,000 cells.

Data analysis. Results presented in the study are triplicate values and representative of three or more independent experiments. Gels were scanned on Epson Expression 1600 Photoscanner using Adobe Photoshop 7.0. Statistical significance was analyzed with the computer software GraphPad Prism. Where appropriate, statistical differences were compared by use of ANOVA or Student’s t-test for unpaired observations. The values presented are means ± SE, and P < 0.05 was considered to be significant.

RESULTS

Chronic and acute exposure of high glucose induces dual effects on cell growth. To determine the effect of chronic (continuous) and acute (10 min) exposure of high glucose on DNA synthesis, we measured [3H]thymidine incorporation into DNA at different time points (12, 24, 36, and 48 h). Figure 1 demonstrates that at 12 h, both chronic (Fig. 1A) and acute (Fig. 1B) exposure of cells with high glucose induced a twofold increase in [3H]thymidine incorporation to DNA. Interestingly, the [3H]thymidine incorporation gradually decreased at 24, 36, and 48 h. At 48 h, there was a 30% reduction in [3H]thymidine incorporation compared with cells exposed to normal concentration of glucose. In support of this finding, we observed a twofold increase in cell number at 12 h, followed by a decrease at 24 h and later time periods (Fig. 1C). The decrease in cell number suggests that high glucose at later time points may induce cellular injury leading to apoptosis and/or cell death. To determine this, we measured the Hoechst 33258 staining of apoptotic cells, which estimates chromatin condensation and nuclear fragmentation at 48 h following chronic and acute exposure of cells to high glucose. Figure 2A demonstrates that cells exposed to normal glucose showed homogeneous staining of their nuclei (1 and 3); in contrast, cells treated with high glucose showed irregular staining of their nuclei (chronic, 2 and acute, 4). Consistent with this finding, we observed a time-dependent increase in the number of dead cells (Fig. 2B).

To determine further whether high glucose-induced cell death involved apoptosis, early apoptosis was examined at 48 h using flow cytometry after double staining the cells with annexin V and PI. In this assay, we determined the ability of the FITC conjugate of annexin V to stain high glucose-exposed cells independently (during early apoptosis) and in combination with PI (during secondary necrosis). The distribution of stained cells is shown in Fig. 3, A and B. In each of the graphs the bottom left quadrant represents live cells, the bottom right quadrant represents cells in early apoptosis, the top right quadrant represents cells in secondary necrosis, and the top left...
quadrant represents cells stained with PI alone. Under high glucose-exposed conditions (chronic and acute), the percentage of cells in early apoptosis was significantly increased compared with normal-glucose exposure. To rule out that the observed effects of high glucose are not an epiphenomenon, we determined the dose-dependent effect of glucose on apoptosis. Cells were exposed (acute) to increasing concentrations (5.5, 10, 15, 20, and 25 mM glucose) and apoptosis was measured at 48 h. Figure 4 shows a significant apoptotic effect at concentrations between 15 and 25 mM glucose, consistent with previous reports of hyperglycemia-induced effects (34, 49, 52, 53). In addition, the effect of 20 mM L-glucose (negative control) and 20 mM mannitol (to induce osmotic stress similar to high glucose) in media containing 5.5 mM D-glucose was measured (17, 30, 32). Figure 5 shows that both agents were ineffective in eliciting apoptosis at 48 h in contrast to D-glucose, which was effective. These results demonstrate that chronic and acute exposure of hPTECs to high glucose has a dual effect on hPTEC involving both proliferation and apoptosis.

Acute exposure of cells to high-glucose levels stimulates time-dependent activation and nuclear translocation of p42/p44 MAP kinases. The observation that the acute and chronic exposure of cells to high glucose can induce analogous effects on cell proliferation and apoptosis suggests that similar intracellular mechanisms may govern these dual but distinct end responses. It has been shown that activation of p42/p44 MAP kinases plays a major role in cell proliferation (9, 48). To elucidate the involvement of the MAP kinase pathway in high glucose-induced cell proliferation and apoptosis, we first examined the time course of p42/44 MAP kinase activation, following a 10-min exposure with high glucose. Figure 6A shows that high glucose activated both forms of MAP kinases in a time-dependent manner, with initial activation observed at 15 min on stimulation (lane 2), and this was still detected at 3 h (Fig. 6A, lane 6). To ensure equal loading of proteins in all lanes, we stripped the blot (Fig. 6A) and reprobed with anti-MAP kinase antibody (Fig. 6A, bottom). To determine whether the activated MAP kinases are translocated to the nucleus.
activated by high glucose (10 min) suggests that they are likely to lead to phosphorylation and nuclear translocation of NF-κB in response to acute exposure of hPTEC to high glucose.

Inhibitors of MAP kinase kinase 1 and NF-κB activation block high glucose-induced cell proliferation in hPTEC. Having demonstrated the activation of p42/p44 MAP kinases and NF-κB in response to acute glucose exposure of hPTEC, we determined whether these two pathways have a role in glucose-induced cell proliferation. To address this, we first examined the ability of NF-κB activation inhibitor and MAP kinase kinase 1 (MAPKK1) inhibitor PD-98059 to inhibit high glucose-induced activation and nuclear translocation of NF-κB and p42/p44 MAP kinases in hPTEC. Figure 8A, top, shows that pretreatment of cells with NF-κB activation inhibitor (10 nM) significantly inhibited high glucose-induced activation of IkB-α in response to acute high-glucose exposure starts at 15 min and remains phosphorylated for the duration of the experiment. Consistent with Ik-B-α phosphorylation, the NF-κB p65 subunit was activated and translocated to the nucleus as early as 20 min on stimulation (Fig. 7B, top). Interestingly, the NF-κB protein remained detectable for the duration of the experiment (Fig. 7B, bottom); however, the phosphorylation of the p65 subunit was significantly reduced after 6 h in the nucleus (Fig. 7B, top). These results suggest that activation of Ik-B-α leads to phosphorylation and nuclear translocation of NF-κB in response to acute exposure of hPTEC to high glucose.

Fig. 5. L-Glucose or mannitol failed to induce apoptosis. Serum-starved (0.5% FBS) cells were exposed to NG or HG (acute). As a negative control, mannitol (20 mM) or L-glucose (20 mM) was added to cells containing 5.5 mM normal Ω-glucose. At 48 h, cells were harvested and stained with FITC-conjugated annexin V and PI for 15 min. The cells were analyzed by a flow cytometer as described in MATERIALS AND METHODS. Results shown are representative of 3 independent experiments.

Following acute high-glucose stimulation (10 min), nuclear extracts were prepared and immunoblotted with phospho-specific anti-MAP kinase antibody. Figure 6B demonstrates that within the first 15 min of stimulation, both p42/p44 MAP kinases were activated and translocated to the nucleus. However, only the p42 MAP kinase showed sustained activation at 3 h. To ensure equal loading of proteins in all lanes, we stripped the blot (Fig. 6B) and reprobed with anti-E2F-2 antibody. E2F-2 is a nuclear localized transcription factor. Figure 6B, bottom, shows that all lanes contained equal amounts of nuclear proteins. These results demonstrate that an initial 10-min exposure of cells with high-glucose concentrations alone can cause sustained activation and nuclear translocation of p42/44 MAP kinases. Although previous investigators have reported the activation of p42/p44 MAP kinases by high glucose in their studies, glucose was continuously present in the medium during the entire time period (13, 42). The detection of MAP kinases nuclear translocation in cells treated with high glucose (10 min) suggests that they are likely to activate nuclear proteins, including transcription factors.

Acute exposure of cells to high glucose stimulates time-dependent activation of Ik-B-α and nuclear translocation of NF-κB. NF-κB, a heterodimer of p65 (Rel A) and p50 proteins, is a transcription factor located in the cytoplasm, where it is bound to the inhibitor κB (IkB-α) (15, 25, 36). It has been shown that during activation of intracellular signal transduction pathway by proinflammatory cytokines, phosphorylation and ubiquitination of IkB occurs, which liberates NF-κB from IkB binding and allows its activation. Because MAP kinases are known to regulate IkB-α through phosphorylation (15), we investigated whether IkB-α is phosphorylated in cells exposed to high glucose for 10 min. Figure 7A, top, shows the time course of activation of IkB-α in response to acute high-glucose exposure starts at 15 min and remains phosphorylated for the duration of the experiment. Consistent with IkB-α phosphorylation, the NF-κB p65 subunit was activated and translocated to the nucleus as early as 20 min on stimulation (Fig. 7B, top). Interestingly, the NF-κB protein remained detectable for the duration of the experiment (Fig. 7B, bottom); however, the phosphorylation of the p65 subunit was significantly reduced after 6 h in the nucleus (Fig. 7B, top). These results suggest that activation of IkB-α leads to phosphorylation and nuclear translocation of NF-κB in response to acute exposure of hPTEC to high glucose.
abolished glucose-induced [3H]thymidine incorporation at all time points (12, 24, 36, and 48 h). Our results show that when high glucose-mediated proliferative stimulus was absent (at later time points), the degree of inhibition observed with inhibitors was more pronounced. We chose 50 μM PD-98059 in these studies because this concentration effectively inhibited high glucose-induced [3H]thymidine incorporation to the level observed with normal glucose in the absence of the inhibitor (Fig. 9D). This suggests that the observed inhibitory effect by 50 μM PD-98059 is not due to cytotoxicity. These results demonstrate that p42/p44 MAP kinases acting through the NF-κB-dependent signaling pathway is responsible for the high glucose-induced proliferation in these cells. To further determine the role of MAP kinases and NF-κB pathways in high glucose-mediated apoptosis, cells were pre-exposed to inhibitors and high glucose-mediated annexin V binding in combination with PI staining measured with flow cytometry. Figure 10 shows that MAP kinase or NF-κB inhibitors failed to reverse high glucose-induced apoptosis, suggesting that these signaling pathways do not contribute to high glucose-mediated apoptosis in these cells. High glucose-mediated inhibition of cell growth is due to the release of an inhibitory factor(s). We hypothesized that high glucose may induce the synthesis and secretion of a factor(s) into the medium, which may be responsible for the observed inhibitory effect on DNA synthesis at 24 h and after. We collected conditioned medium from high glucose-treated cells, which had been exposed to high-glucose medium for the first 10 min and subsequently incubated with normal-glucose medium for 24 h. We then examined with fresh cells whether the

![Image](http://ajprenal.physiology.org/)
conditioned medium would cause the inhibition of high glucose-induced incorporation of [3H]thymidine in fresh cells at 12 h. In these experiments, we chose 12 h as the end point to measure [3H]thymidine incorporation, because our previous experiments had shown that glucose-induced maximal DNA synthesis occurs at 12 h (Fig. 1). Figure 11 shows that conditioned medium from cells treated with high glucose (acute) significantly inhibited high glucose-induced [3H]thymidine incorporation into DNA in fresh cells at 12 h. In contrast, conditioned medium from cells treated with no glucose or normal glucose had no significant inhibitory effect on DNA synthesis at 12 h. These results show that the conditioned medium collected from cells exposed to high glucose contains an inhibitory factor(s), and it may be responsible for the observed decrease in DNA synthesis at 24 h and longer.

**Acute exposure to high glucose induces ROS and promotes apoptosis in hPTEC.** Chronically exposed to cells high glucose has been reported to alter the redox status of cells, through overproduction of ROS (38, 41), and ROS has been shown to promote oxidant-induced apoptosis (1). Therefore, we determined whether an acute (10 min) exposure of hPTEC with high glucose could generate ROS similar to chronic exposure. Serum-starved cells were treated initially with high glucose for 10 min, washed, incubated with normal glucose for 12 and 48 h, and ROS was measured using flow cytometry. hPTEC acutely exposed to high glucose demonstrates that at 12 h, there is no increase; however, there is a significant increase in ROS activity at 48 h (Fig. 12, 2 and 4). These results are consistent with the observed effects in cells chronically exposed to high glucose (Fig. 12, 3 and 4). As a positive control, cells were treated with the oxidative stress-inducing agent, buthionine sulfoxamine (BSO), and ROS activity was measured (Fig. 12, 1). Pretreatment of cells with N-acetyl L-cysteine (NAC), a well-known thiol antioxidant, reversed high glucose (acute or chronic)-mediated ROS production in hPTEC (Fig. 12, 2 and 3). We extended these experiments to include the effect of antioxidant NAC to reverse the high glucose-induced apoptosis at 48 h. Annexin V binding in combination with PI staining was measured using flow cytometry to determine whether early apoptosis in cells treated with high glucose (acute/chronic) occurred. Figure 13 shows that high glucose failed to induce apoptosis in cells preexposed to antioxidant NAC. These results demonstrate that acute exposure of hPTEC to high glucose is capable of inducing not only ROS but is also responsible for the observed apoptotic effect.

**DISCUSSION**

The development of diabetic nephropathy is associated with chronic exposure of renal cells to high glucose (6, 47, 58). From a pathophysiological point of view, patients undergoing therapy are potentially exposed to transient increases in plasma glucose levels before treatment (such as insulin administration). In fact, studies have shown that a significant number of these patients under treatment also develop diabetic nephropathy (7, 35, 43). Therefore, it is imperative to consider the possibility that such transient increases in plasma glucose could result in elevated glucose levels in renal tubular secretions. This may contribute to proximal tubule cell damage, leading to epithelial cell dysfunction. Because overwhelming evidence indicates a role for long-term hyperglycemia in the
pathogenesis of diabetic nephropathy, much effort has been
directed toward identifying the chronic effects of high glucose
on cell damage, and very little attention has been given to the
acute effects of glucose. In this study, we demonstrate for the
first time that acute (10 min) exposure of hTPEC with high
sugar induces a time-dependent dual effect, with an initial
proliferative phase occurring via activation of p42/p44 MAP
kinases and NF-kB, whereas the apoptotic effect requires the
secretion of an inhibitory factor into the medium 24 h after acute
high-glucose exposure. The capacity of short-term exposure
of high glucose to induce both cell proliferation and apoptosis
suggests that a transient increase in extracellular glucose can
contribute to the cellular dysfunction in early diabetic nephro-
apathy. We believe that this represents an important observation
within the context of effective diabetic management.

Independent studies conducted in different cell types have
demonstrated cell-specific effect of chronic exposure of high
sugar on cell proliferation or apoptosis. For example, in
proximal tubule cells, exposure to high glucose for 24 h or
more was shown to inhibit DNA synthesis (41). In another
study on proximal tubular epithelial cells, Allen et al. (1)
showed that high-glucose treatment for 24–48 h induced
oxidative stress followed by apoptosis. In mesangial cells,
high-glucose exposure (24–48 h) caused an increase in
[3H]thymidine incorporation and cell number; however, the
effect was normalized at 72 h (40). In renal cortical fibroblasts,
high-glucose exposure (24–72 h) significantly increased
[3H]thymidine incorporation and cell number (19). Although
these studies have documented the proliferative or apoptotic
effects of chronic high glucose in different renal cell types, to
date, there is no report on the ability of high glucose (acute or
chronic) to induce both responses in the same cell type.
Therefore, our result in hPTEC that acute high glucose can

Fig. 10. Effect of PD-98059 and NF-kB on HG (acute)-induced apoptosis. Serum-starved (0.5% FBS)
cells were left untreated or pretreated with PD-98059 or NF-kB inhibitor and exposed to HG (25 mM) for 10
min. Cells were washed with PBS, following which, medium containing NG was added and incubated. At
48 h, cells were harvested and stained with FITC-conjugated annexin V and PI for 15 min. The cells
were analyzed by a flow cytometer as described in MATERIALS AND METHODS. Results are from 1 of 3
representative experiments.

Fig. 11. Effect of conditioned medium from HG-exposed (acute) cells on
DNA synthesis. Serum-starved (0.5% FBS) cells were treated for 10 min,
washed with PBS, and incubated with NG medium for 24 h before conditioned
medium was collected. Fresh cells grown in normal medium were exposed to
a mixture of normal and conditioned medium (2:1) for 12 h. [3H]thymidine
incorporation was measured as an indicator of DNA synthesis as described in
Fig. 1. Data are expressed as means ± SE of triplicate. *P < 0.05 compared
with cells exposed to no glucose or NG.
induce a time-dependent proliferative and apoptotic effect is a significant observation.

We demonstrated that acute (10 min) exposure of hPTEC to high glucose induced maximal \[^3H\]thymidine incorporation at 12 h following treatment. Interestingly, we also observed that treatment of cells to high glucose for 2.5 and 5 min also induced a time-dependent effect (Fig. 14). To gain insight into the mechanisms of acute effects of high glucose on cell proliferation, we studied glucose-induced activation of p42/p44 MAP kinases and NF-κB. Several studies have shown that p42/p44 MAP kinases play an important role in cell proliferation (9, 48), and an anti-apoptotic role has been demonstrated for NF-κB (26, 36). Depending on the duration of activation, MAP kinases may reside in the cytoplasm or get translocated to the nucleus (9). Our findings demonstrate that acute exposure of cells to high glucose can induce sustained activation of p42/p44 MAP kinases and NF-κB and causes their nuclear translocation. In addition, inhibition of the pathway by the MAPKK1 inhibitor prevented glucose-mediated activation of NF-κB. This suggests that NF-κB is a downstream intermediate to p42/p44 MAP kinases in glucose-induced signal transduction. Treatment of cells with a MAPKK1 inhibitor or NF-κB activator inhibitor abolished glucose-mediated proliferative effects. However, these inhibitors failed to prevent glucose-mediated apoptosis. This demonstrates that both p42/p44 MAP kinases and NF-κB are important regulators of glucose-mediated cell proliferation in hPTEC.

In addition to cell proliferation at 12 h, we observed that at 24 h or later, acute high-glucose exposure caused inhibition in \[^3H\]thymidine incorporation/cell number and induced apoptosis. The inhibition in DNA synthesis at 24 h or later does not appear to be related to overconfluency of cell growth. This is supported by the observation that conditioned medium from cells treated with high glucose (10 min) effectively inhibited high glucose-mediated DNA synthesis in freshly grown cells (75% confluency) at 12 h, the time point at which maximum DNA synthesis is expected to occur. Apoptosis is a closely regulated event controlled by genetic programming. Identifying the pathways and mechanism(s), which initiate these events, is important because of the end effect on renal cell damage. Although p42/p44 MAP kinases and NF-κB are not involved in high glucose-mediated apoptosis, it appears to...
require the release of a secondary factor into the conditioned medium. In this context, it is important to note that other investigators have reported that chronic exposure to high glucose can induce the secretion of TGF-β into the conditioned medium in tubular epithelial, glomerular mesangial, and renal cortical fibroblast cells (19, 45, 59). However, in preliminary studies, we observed that direct exposure of hPTEC to TGF-β failed to elicit inhibition of high glucose-induced [3H]thymidine incorporation, suggesting that TGF-β is not involved. The identification of the factor(s) released by hPTEC in response to high glucose, which mediates inhibition of DNA synthesis, requires further study.

Several lines of evidence demonstrate that oxidative stress or ROS is involved in diabetes-induced renal complications (1, 31). Moreover, it was shown that antioxidant therapy prevents the development of kidney disease in animal models of diabetes (28, 29), and experimental evidence obtained from in vitro studies shows that prevention of ROS generation defends...
against the damaging effects of hyperglycemia on mesangial cell function (50, 51). In our study, we observed no induction of ROS during the early proliferative phase (12 h). However, a significant induction of ROS was observed 24 h after acute exposure of cells to high glucose. The delayed appearance of ROS observed in hPTEC in response to high glucose is consistent with the previous reports of ROS production in other cells (20, 25). Importantly, the incubation of cells with the antioxidant precursor NAC significantly reversed the high glucose-mediated ROS generation and subsequent induction of apoptosis. These observations show that ROS is involved in glucose-mediated (acute) hPTEC cell damage. In some studies, it was shown that ROS generation by chronic high-glucose exposure causes the production of advanced glycation end products (AGEs), which then can also contribute to apoptosis (47, 57). AGEs are generated when glucose and other reactive carbonyl compounds react nonenzymatically with proteins, lipids, or nucleic acids, resulting in the formation of Schiff bases. Some examples of AGEs are carboxymethyl lysine, pentosidine, imidazolone, and pyrraline (2, 47). Whether these AGE products represent inhibitory factors released by high glucose to affect cell damage in hPTEC is currently under investigation.

The overall focus of the study was to determine the effect of acute exposure of high glucose on hPTEC. Cells were exposed for 10 min to high-glucose levels and then replaced with normal-glucose medium to avoid continuous activation of signaling by extracellular glucose. We observed that a 10-min exposure of hPTEC to high glucose alone was sufficient to trigger the signaling intermediates (MAP kinase and NF-κB) in a sustained fashion and also elicited an early proliferative (12 h) and a late apoptotic effect (48–72 h). In these cells, the observed effect of glucose does not appear to be caused by changes in the osmolarity of the medium (normal glucose 315 ± 2.52 and high glucose 334 ± 1.67 mosmol/kgH2O). The increase in osmolarity observed with high glucose is similar to previously reported values in the literature (10) and within the range of commercially available cell culture media. Furthermore, apoptosis was not observed in cells exposed to mannitol-containing medium with similar osmolarity suggesting that the observed effect of α-glucose was not secondary to osmotic load. High glucose-mediated rapid activation of signaling intermediates without sustained stimulation suggests the possibility of a direct role of glucose in the observed effects. Glucose may mediate these effects by coupling through cell surface glucose sensors. For example, in *Saccharomyces cerevisiae*, extracellular glucose utilizes the two transmembrane glucose sensors Snf3 and Rgt2 to activate casein kinase 1 through a G protein-coupled mechanism(s) (37, 44). In mammalian cells, in addition to transport function, the glucose carrier proteins, GLUT1 and GLUT2, were shown to act as glucose sensors to activate p42/p44 MAP kinases (3, 16, 23). In this case, the binding of glucose to these sensors is hypothesized to induce a conformational change leading to the initiation of signaling. In hPTEC, we detected the presence of GLUT1 and GLUT2 (Thekkumkara TJ, unpublished observations), which suggests the possible involvement of these transporters in glucose-mediated signal transduction. In addition, sodium/glucose symporter carrier proteins, which are expressed in the apical plasma membrane portion, may also be involved in the initiation of signaling. Compared with other cell types, in epithelial cells, the apical plasma membrane has a 25-fold increase in cell surface area due to the presence of microvilli, which may significantly increase the number of sodium/glucose symporter protein(s). Their increased presence on the apical membrane may allow for the transport of a significant amount of extracellular glucose into the cell within a short period of time. Therefore, the role of the sodium/glucose symport system in the initiation of signaling pathways cannot be ruled out.

In summary, we demonstrated that acute exposure of cells to high glucose activates intracellular signaling intermediates, p42/p44 MAP kinases and NF-κB, and stimulates a time-dependent dual effect involving cell proliferation and apoptosis. Although these cells were grown in normal-glucose medium, a transient increase in extracellular glucose by a single dose induced a sudden change in cell signaling, leading to long-term alterations in cellular functions. Surprisingly, these effects of acute high-glucose exposure are analogous to the previously observed cellular effects with chronic high-glucose exposure. This implies that acute exposure of cells to high glucose can contribute to renal cellular injury similar to that which is observed in hyperglycemia. Furthermore, our results highlight the notion that optimal management of diabetic complications will require a full understanding of the interplay between acute and chronic effects of hyperglycemia.

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REFERENCES

GLUCOSE-INDUCED DUAL EFFECTS IN PROXIMAL TUBULE CELLS


