Intermittent high glucose enhances apoptosis in human umbilical vein endothelial cells in culture

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Intermittent glucose; endothelium; apoptosis; Bcl-2 protein; Bax protein

APOPTOSIS, OR PROGRAMMED CELL DEATH, is an active process of cell suicide morphologically and biochemically different from necrosis and is actively regulated (17). Several lines of evidence suggest that endothelial dysfunction and damage present early steps in the pathophysiology of vascular complications in diabetes mellitus (10, 19). Hyperglycemia is the central initiating factor for all types of diabetic microvascular disease (24). Studies using cultured endothelial cells clearly show that incubation of these cells with a high concentration of glucose leads to severe changes in proliferation and in the adhesive and synthetic properties of the cells (20). Thus an accumulation of partly damaged and dysfunctional cells has to be expected in the vasculature in diabetic conditions, which could rapidly lead to severe vascular defects in the regulation of endothelial function. Endothelial cell death and loss of functional endothelium are associated with a reduced thrombore-

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(GIBCO BRL, Gaithersburg, MD) supplemented with 2 mmol/l glutamine (GIBCO), 20% heat-inactivated fetal calf serum (GIBCO), 25 μg/ml endothelial cell growth supplement, 90 μg/ml heparin (GIBCO), 50 U/ml penicillin, 50 U/ml streptomycin (GIBCO), and 0.25 μg/ml fungizone (GIBCO). The Petri dishes were incubated at 37°C in a 5% CO2-95% air gas mixture. Primary cultures were fluid changed 24 h after seeding and were subcultured on reaching confluence by use of 0.01% trypsin-EDTA, and the trypsin was inactivated by dilution. Cultured cells were identified as endothelial by their morphology and the presence of factor VIII-related antigen detected using indirect immunofluorescence. The primary antibody was rabbit anti-human von Willebrand factor (1:40 dilution, Dako), and the secondary antibody was GIBCO BRL Premium Quality FITC-conjugated goat anti-rabbit IgG (1:50 dilution). Only first- and second-passage HUVECs were used in the study to avoid "age-dependent" variation in levels of apoptosis (30). HUVECs were seeded at equal density (1.3 × 10^6 in gelatin-coated 60-mm Petri dishes) and allowed to attach overnight; then they were exposed to the experimental conditions for 14 days. Therefore, three groups of cells were formed, each one receiving the following fresh media every 24 h, respectively: 1) continuous normal glucose medium (5 mmol/l), 2) continuous high-glucose medium (20 mmol/l), and 3) alternating normal and high-glucose media every 24 h. Osmotic control was assured by incubating cells with 20 mmol/l mannitol, both continuously or in alternating fashion.

**HUVEC Apoptosis Assay**

Viability assay. HUVECs were trypsinized, washed, and stained with PBS containing 20 μg/ml (final concentration) of propidium iodide with Facscan (Becton-Dickinson, Mansfield, MA) equipped with a 15-MW argon-ion laser and the CellQuest analysis software. The percentage of permeabilized cells was evaluated by cytofluorometric analysis.

**Cell cycle analysis.** DNA content of the cell populations was determined by flow cytometry. Briefly, monolayers of HUVECs were washed with PBS solution and stained for 1 h with 50 μg/ml of propidium iodide in 0.2% sodium citrate containing 12.5 μg/ml of RNase. Then the cells were harvested from Petri dishes by gentle scraping and analyzed with the cytofluorimeter. Histograms of cell number vs. linear integrated area of the fluorescence pulse (FL2-A) were recorded for 30,000 nuclei at flow rates no higher than 50–100 events/s. Cytograms of FL2-W (width of fluorescence pulse) vs. linear FL2-A were used to gate out aggregates and cell debris. The histograms were analyzed with the Modfit 2.0 software according to guidelines proposed to Duque et al. (13).

**Morphological analysis.** The cells grown on gelatin-coated slides were washed with PBS, fixed with a freshly prepared mixture of 5% acetic acid-95% ethanol, washed with PBS, and treated with 0.5 μg/ml of DAPI (4,2-diamidino-2-phenylindole) for 30 min. The slides were examined under a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an ultraviolet (UV) filter BP-330–385.

**DNA fragmentation.** ELISA. An ELISA kit (Boehringer Mannheim, Mannheim, Germany) which quantitatively detects cytosolic histone-associated DNA fragments, was employed to assess apoptosis in adhered HUVECs. HUVEC DNA fragments were measured according to the procedures described in the ELISA kit. Briefly, the cytosolic fraction (13,000-g supernatant) of HUVECs was used as antigen source in a sandwich ELISA with a primary anti-histone monoclonal antibody coated to the microtiter plate and a second anti-DNA monoclonal antibody coupled to peroxidase. The percentage of DNA fragmentation was calculated according to the following formula: % DNA fragments = [(OD-stimulated cells – OD blank)/OD control – OD blank] × 100, where OD is optical density.

**DNA gel electrophoresis.** For qualitative determination of apoptosis by DNA fragmentation, gel electrophoresis for HUVEC DNA was carried out according to a modified procedure for assaying DNA fragmentation in total genomic DNA (31). Briefly, HUVECs were washed twice with Hanks’ balanced salt solution and pelleted by centrifugation at 250 g for 5 min. The cell pellets were resuspended with 20 μl of lysis buffer (pH 8.0, 20 mM EDTA, 100 mM Tris, and 0.8% N-lauroylsarcosine) and 10 μl of RNase A (pH 4.8, 1 mg/ml containing 100 mM sodium acetate and 0.3 mM EDTA) for 6 h at 37°C in a water bath. After treatment with 10 μl proteinase K (20 mg/ml) overnight at 50°C in a water bath, the DNA preparations were added to 5 μl of loading buffer (10 mM EDTA, 0.25% bromophenol blue, and 50% glycerol) and were electrophoresed on a 1.5% agarose gel containing 0.3 μg/ml ethidium bromide in TBE buffer (pH 8.0, in mM: 2 EDTA, 89 Tris, and 89 boric acid) for 3 h. HaeIII digest of φX174 DNA was applied to each gel to provide size markers of 1.4-, 1.1-, 0.9-, 0.6-, and 0.3-kb pairs, respectively. Gels were photographed under UV transillumination.

**Bel-2 and Bax expression.** Experiments were performed by Western blotting analysis with specific antibodies directed against either the anti-apoptotic protein Bel-2 or the proapoptotic protein Bax (22). Cells were washed three times in cold PBS and then lysed for 30 min at 4°C in buffer containing 1% Nonidet P-40 and (in mM) 50 Tris-HCl (pH 7.5), 100 NaCl, 5 EDTA, and 1 phenylmethylsulfonyl fluoride. After centrifugation at 13,000 g for 10 min at 4°C, the supernatant was collected, and the protein content of all samples was determined using the Bradford assay (7), with bovin serum albumin as a standard.
To perform Western blot analysis, 20 μg of HUVEC protein lysate were boiled in Laemmli sample buffer and resolved by 12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK), blocked for 2 h in 5% nonfat dry milk in TBS (50 mmol/l Tris·HCl, pH 7.5, and 150 mmol/l NaCl), and washed with TBS+0.1% Tween 20, and the incubate at room temperature for 2.5 h with mouse anti-human Bcl-2 or anti-human Bax antibodies (5 μg/ml). The filter was later washed with TBS+0 and 1% Tween 20, and detection was performed using a secondary horseradish peroxidase-linked anti-mouse antibody (1:7,000), an enhanced chemiluminescence system (Amersham), and exposure to Kodak Bio Max Light-1 films. The intensity of the Western blot signals was quantified by densitometry.

**Statistical Analysis**

All data are means ± SD. Groups were compared using analysis of variance, and the Bonferroni-Dunn post hoc test.
RESULTS

The results of six different experiments for each experimental condition were analyzed through a 14-day period.

Cell Viability and Apoptosis

Significant variations of viability and cell cycle distribution were observed starting at day 7 and were further enhanced at day 14. As shown in Fig. 1, after 7 days, viability of HUVECs was significantly affected by fluctuating glucose condition (P < 0.05 vs. both normal and stable high glucose). After 14 days, stable high glucose significantly affected cell viability (P < 0.05 vs. normal glucose). However, at this time, fluctuating glucose produced a significant increase of cell death compared with both normal and stable glucose concentrations (P < 0.01; Fig. 1). Nonspecific effects due to osmolarity changes can be ruled out, because the cytotoxic effect was not detected in the cells cultured in the presence of 20 mmol/l mannitol, either continuously or in alternating fashion (data not shown).

In agreement with the above observation, a significant number of apoptotic cells was observed in cell populations cultured with fluctuating glucose for 14 days. The analysis of DNA content showed that stable high glucose induced an increase in the percentage of apoptotic cells represented by a sub-G0-G1 (P < 0.05) peak compared with normal glucose concentration. Intermittent high glucose enhanced this effect (P < 0.01 vs. both normal and stable high glucose; Fig. 2, A and B). A striking decrease in phase S cells (P < 0.01) was detected in cultures treated with a steady high glucose compared with normal glucose (Fig. 2, A and C). Intermittent high glucose produced a worse effect (P < 0.01 vs. stable high glucose, P < 0.001 vs. normal glucose; Fig. 2, A and C). A delay in cell cycle progression has been reported in HUVEC cells cultured in high glucose (13). Our data confirm these results and show a significant magnification of cell cycle delay associated with fluctuating doses of glucose supply.

Apoptotic death was monitored also by a morphological analysis, showing after 14 days a pronounced nuclear piknosis in HUVEC populations treated with fluctuating glucose (Fig. 3). At the 14th day, ELISA showed that stable high glucose caused increased DNA fragmentation when compared with normal glucose (P < 0.01) and that intermittent high glucose had a more deleterious effect (P < 0.01 vs. stable high glucose and P < 0.001 vs. normal glucose; Fig. 4). DNA gel electrophoresis confirmed this evidence (Fig. 5). There was no increase in DNA fragments in the mannitol-treated groups.

Expression of Bcl-2 and Bax Proteins

Representative Western blotting of Bcl-2 and Bax is reported in Figs. 6 and 7. The Western blotting analysis of electrophoresed proteins present in lysed cells showed that, after 7-day culture, Bcl-2 protein was significantly decreased in the fluctuating condition compared with either normal or stable high glucose (P < 0.05; Figs. 6 and 8). After 14 days, Bcl-2 was present at a significantly lower level in the cells cul-
tured in the presence of 20 mmol/l glucose compared with cells cultured in 5 mmol/l glucose (%P, 0.05; Figs. 6 and 8). In cells exposed to fluctuating concentrations of glucose, Bcl-2 protein was almost undetectable (%P, 0.01 vs. stable high glucose, %P, 0.001 vs. normal glucose; Figs. 6 and 8).

Bax expression was the opposite of Bcl-2: after 7 days it was significantly increased in the fluctuating condition compared with both normal and stable high glucose conditions (%P, 0.01); after 14 days it was significantly increased in cells cultured in stable glucose compared with normal glucose (%P, 0.01) and significantly enhanced in the fluctuating condition compared with both normal (%P, 0.001) and stable high glucose (%P, 0.01; Fig. 7 and 8) conditions.

**DISCUSSION**

The studies performed show that both intermittent and high glucose concentrations stimulate apoptosis; however, intermittent glucose concentrations appear to worsen the proapoptotic effects of high glucose on HUVECs. These findings suggest that variability in glycemic control could be more deleterious to HUVECs than a constant high concentration of glucose.

Evidence for apoptosis has been obtained by several methods. Furthermore, the expression of the proteins Bcl-2 and Bax, which are involved in the apoptotic process (22), has been evaluated. All methods confirm that intermittent high glucose is more effective than stable high glucose in producing HUVEC apoptosis. Data published by Lorenzi et al. (20) show that high glucose interferes with the regulation of the cell cycle. Baumgartner-Parzer et al. (6) suggested that high glucose retards cell cycle transition from the S and G2 phases, favoring cell

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**Fig. 4.** DNA fragmentation of HUVECs by ELISA. Values are means ± SD of 6 separate experiments. %P < 0.01 vs. glucose 5 mmol/l; %P < 0.01 vs. glucose 20 mmol/l; %P < 0.001 vs. glucose 5 mmol/l.

**Fig. 5.** Gel electrophoresis for detection of HUVEC DNA fragmentation. Lane 1: DNA size markers. Lanes 2–5: mannitol; 5 mmol/l glucose; 20 mmol/l glucose; 5/20 mmol/l glucose.

**Fig. 6.** Western blot of Bcl-2 after 14 days of experiment. Lane 1: negative control; lane 2: 5/20 mmol/l glucose; lane 3: 20 mmol/l glucose; lane 4: 5 mmol/l glucose.

**Fig. 7.** Western blot of Bax after 14 days of experiment. Lane 1: 5/20 mmol/l glucose; lane 2: 20 mmol/l glucose; lane 3: 5 mmol/l glucose; lane 4: negative control.
death. Our data confirm this phenomenon in stable high glucose, showing, furthermore, that it is amplified in the fluctuating glucose condition.

Within the complex network of proteins regulating apoptosis, proteins of the Bcl-2 family are recognized to play a central role (2). Members within this wide family have been classified into prosurvival or antiapoptotic proteins, Bcl-2, and proapoptotic proteins, Bax (2). Analyses of the level of expression of these two proteins during a period of primary culture reveal time-dependent changes that correlate with the changes in the susceptibility of cells to undergo apoptosis (2). The highest levels of Bcl-2 are observed during the early times of culture, when cells have a low propensity to undergo apoptosis (2). The progressive downregulation of Bcl-2 and the increased expression of Bax during the period of culture account for the variations in the apoptosis status of the cells (2).

In our experimental system, apoptosis is associated with a remarkably decreased expression of the Bcl-2 protein and with a simultaneous increase of Bax. It has been shown that Bcl-2 also protects endothelial cells from apoptosis (1), whereas it has been reported that high glucose significantly decreases Bcl-2 expression and increases the number of dead cells in the culture of renal proximal tubular epithelial cells (23). In this experiment, mannitol did not reproduce these effects of high glucose (23). In our study, high glucose induces a decrease of Bcl-2 in HUVECs, which is accompanied by an opposite increase of Bax, an effect followed by increased cell apoptosis. This phenomenon is amplified during glucose fluctuations. It seems true that high glucose modulates the expression of apoptosis-regulatory genes in HUVECs so as to favor apoptosis. However, the fluctuation of glucose seems to exert a more dangerous effect.

A proapoptotic effect of acute glucose loss has been reported for pericytes previously cultured in high glucose (18). This finding has led to the hypothesis that preexisting hyperglycemia primes the apoptotic responsiveness of pericytes (18). On this basis, it should be hypothesized that, in HUVECs, the adverse effects due to frequent fluctuations from high to low-normal glucose level may accumulate through the study, producing the phenomenon observed in our study.

A deleterious effect of glucose fluctuations for both mesangial (26) and tubulointerstitial cells (22) has been reported. Mesangial cells cultured in periodic high glucose concentration increase matrix production more than cells cultured in high stable glucose (16). Similarly, fluctuations of glucose display a more dangerous effect than stable high glucose on tubulointerstitial cells, in terms of collagen synthesis and cell growth (16). Consistently, our data also show a deleterious effect of fluctuating glucose for HUVECs. Thus cumulating in vitro evidence suggests that glucose variations may be more dangerous for the cells than stable high glucose.

At present, the molecular mechanisms triggered on cultured HUVECs by periodically changing glucose concentrations are not known. Elevated extracellular glucose concentration has several effects on the intracellular signaling pathway, including increases in diacylglycerol content (4), protein kinase C activation (4), oxidative stress generation (9), activation of calcium channels (25), and changes in cellular myoinositol (14), glycosphingolipid (33), and prostaglandin content (11). Because these metabolic changes may trigger or may be related to apoptosis (3, 32), they could be involved in death caused by glucose fluctuations. During chronic exposure to high glucose, some metabolic variations induced by this constant situation might change or feed back regulatory cell controls, partially counteracting the toxic glucose effect. Intermittent exposure to high glucose might reduce such adaptation, causing more pronounced toxicity.

Our findings have potential clinical relevance. It is now recognized that both hyperglycemia at 2 h of glycemia during an oral glucose challenge (5, 27) and glucose fluctuation (21) per se are strong predictors of cardiovascular disease. Because it has been suggested that these “hyperglycaemic spikes” may play a significant role in the pathogenesis of vascular diabetic complications (8), our data support the hypothesis that improved clinical outcomes may be related to the effort to decrease glucose fluctuations in diabetic patients.

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Fig. 8. Time course of Bcl-2 and Bax proteins expressed as optical density units measured by Western blot analysis. Values are means ± SD of 6 separate experiments. *P < 0.05 vs. glucose 5 mmol/l; **P < 0.05 vs. glucose 20 mmol/l; $$$P < 0.01 vs. glucose 5 mmol/l; $$$P < 0.001 vs. glucose 5 mmol/l.
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