High glucose augments stress-induced apoptosis in endothelial cells.

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Abstract Hyperglycemia has been identified as one of the important factors involved in the microvascular complications of diabetes, and has been related to increased cardiovascular mortality. Endothelial damage and dysfunction result from diabetes; therefore, the aim of this study was to determine the response of endothelial cells to stressful stimuli, modelled in normal and high glucose concentrations in vitro. EAhy 926 endothelial cells were cultured in 5 mmol/L or 30 mmol/L glucose conditions for a 24 hour period and oxidative stress was induced by exposure to hydrogen peroxide (H2O2) or tumour necrosis factor-α (TNF-α), following which the protective effect of the glucocorticoid dexamethasone was assessed. Apoptosis, necrosis and cell viability were determined using an ELISA for DNA fragmentation, an enzymatic lactate dehydrogenase assay and an MTT assay, respectively. High glucose significantly increased the susceptibility of EAhy 926 cells to apoptosis in the presence of 500 μmol/L H2O2, above that induced in normal glucose (P<0.02). A reduction of H2O2- and TNF-α-induced apoptosis occurred in both high and low glucose after treatment with dexamethasone (P<0.05). Conclusion high glucose is effective in significantly augmenting stress caused by H2O2, but not in causing stress alone. These findings suggest a mechanism by which short term hyperglycemia may facilitate and augment endothelial damage. (J Geriatr Cardiol 2009; 6:102-107)

Key words Hyperglycemia; endothelial cells; oxidative stress; glucocorticoid

Introduction

Glycemic control has been shown to be important in the development of the microvascular complications of diabetes.1,2 However, people with diabetes are also at increased risk of macrovascular disease such as coronary heart disease and stroke,3 and it has recently been shown that hyperglycemia based on HbA1c is related to increased cardiovascular mortality.4 The results of the Diabetes Intervention Study indicate that postprandial hyperglycemia, but not fasting hyperglycemia, is an independent risk factor for myocardial infarction and total mortality in newly detected non-insulin-dependent diabetes mellitus.5 In addition, glycemic excursion through postprandial hyperglycemia has been suggested to be important in the development of diabetic complications.6

The effects of hyperglycemia may be mediated by an increase in the production of free radicals, leading to oxidative stress.7 Glucose is prone to autoxidation, generating H2O2 and oxygen-derived free radicals that cause structural alterations and damage to lips and proteins.8 Endothelial cells are particularly prone to oxidative stress, owing to their location and functions and a number of in vitro effects of high glucose on cultured human endothelial cells have been reported. These include, triggering of apoptosis,9 lengthening of proliferation time,10,11 enhanced adherence of monocytes12 and an increase in circulating ICAM-1.13 The reported induction of antioxidant enzymes14 suggested that oxidative stress may have an important role in causing endothelial cell damage in vivo.

Apoptosis and necrosis may both contribute to cell death in cardiovascular disease, ischemia and myocardial infarction.15,16 However, these two forms of cell death differ, with apoptosis being an actively regulated energy requiring process resulting in no surrounding cell damage. Conversely, necrosis is associated with rupture of the cell membrane and release of the intracellular constituents into the surrounding tissue, which initiates an acute inflammatory response.17,18

A number of potential protective mechanisms exist to prevent oxidant-induced damage to endothelial cells, and include antioxidants and glucocorticoids. In addition to their anti-inflammatory and immunosuppressive effects, glucocorticoids have been reported to enhance tissue and cell survival under stressful and damaging conditions, through stabilization of endothelial and lysosomal membranes.19,20 Dexamethasone(dex) has been shown to protect mouse fibroblasts against TNF-α,21 rat intestinal epithelial cells against oxidant injury,22 and to inhibit apoptosis and prolong viability of cultured human neutrophils.23

Obesity is associated with increased production of cytokines, including TNF-α secreted by activated macrophages. Metabolic effects of TNF-α are implicated in the development of insulin resistance in type 2 diabetes,
obesity and aging. TNF-α is cytotoxic to many cell types in vitro, including vascular endothelial cells and several mediators are involved in TNF-α-induced cell death. TNF-α-induced apoptosis is signalled via activation of CPP32-like protease and requires mitochondrial respiratory function.

This study used the immortalized endothelial cell line, EAhy 926, a hybrid between human umbilical vein endothelial cells (HUVECs) and the lung tumor cell line, A549. The aim of the study was to determine whether acute exposure to high glucose concentrations altered the responses of this model to oxidative stress and whether an effect was modified by intervention with the synthetic glucocorticoid dexamethasone.

Methods

Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DMEM), trypsin-EDTA solution, L-glutamine, penicillin-streptomycin, amphotericin B (Fungizone), fetal bovine serum (FBS) and bovine serum albumin (BSA) were obtained from Life Technologies (Paisley, Scotland). All chemicals were purchased from Sigma Aldrich (Poole, UK), unless otherwise stated, and were of analytical grade. NAC was from Calbiochem (Cambridge, UK). The Cell Death Detection ELISA PLUS and Cytotoxicity Detection Kit (LDH) were from Roche (Lewes, UK).

Cell culture

EAhy 926 cells were cultured in 75 cm² flasks (Sarstedt, NC, USA), in DMEM, containing 1,000 mg/L D-glucose, 1 mmol/L sodium pyruvate, 25 mmol/L HEPES, and 4 mg/L pyridoxine and supplemented with 10% (v/v) FBS, L-glutamine (2 mmol/L), penicillin-streptomycin (100 mg/ml) and amphotericin B (2.5 mg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were detached using trypsin-EDTA solution (0.05 g/L trypsin and 0.02 g/L EDTA), centrifuged (1,000 rpm for 3 minutes, at room temperature), distributed (1,000 rpm for 3 minutes, at room temperature), distributed (1,000 rpm for 3 minutes, at room temperature) between supplemented DMEM containing 5 mmol/L glucose or 30 mmol/L glucose and seeded into 48-well culture plates (SLS, Nottingham, UK), at a density of 2 × 10⁴ per well. Cells were allowed to attach for 24 hours prior to the addition of any reagents.

Measurements of apoptosis, necrosis and viability

Apoptotic cell death was measured, 24 hours after addition of H₂O₂ or TNF-α, using a quantitative immunoassay kit, the Cell Death Detection ELISA PLUS (Roche, 1774425), according to the manufacturer’s instructions. The extent of necrotic cell death was determined using the Cytotoxicity Detection Kit (Roche, 1644793), according to the manufacturer’s instructions, to measure the activity of lactate dehydrogenase (LDH). Cell viability was determined by the methylthiazol tetrazolium (MTT) assay, using a modification of the method of Mosmann (Mosmann, 1983). MTT solution (0.1 mg/ml final concentration) was added to the cells and incubated overnight at 37°C. The resulting formazan crystals were dissolved in 1% sodium dodecyl sulphate (SDS) (20% in 20 mmol/L HCl) by incubating for 4-5 hours at 37°C. Absorbance was measured immediately at 540 nm.

Experiments were performed in DMEM containing 5 mmol/L D-glucose or 30 mmol/L D-glucose, and in parallel in 5 mmol/L D-glucose with 25 mmol/L L-glucose, to control for the confounding effects of osmolality. All experiments were performed in triplicate. Measurements of apoptosis and necrosis were taken from the same wells, while measurements of viability were made on separate plates of identically treated cells.

In order to establish that the effect of H₂O₂ in this experimental system was mediated via the induction of oxidative stress, the effects of the antioxidant, N-acetyl cysteine (NAC) and the iron-chelating agent, deferoxamine, were assessed. Deferoxamine renders iron unavailable for the Fenton reaction, by which H₂O₂ forms the hydroxyl radical, effecting cell damage. Cells were treated with either 1 mmol/L NAC or 100 mmol/L deferroxamine for 30 minutes, prior to exposure to a dose range of 300 mmol/L to 900 mM H₂O₂, for 24 hours.

As apoptosis is energy-dependent, an assessment of apoptosis-inhibition by the mitochondrial toxin 3-nitropropionic acid (3-NP), a succinate dehydrogenase inhibitor, was also made. Cells were treated with 2 mmol/L 3-NP for 24 hours, then both 3-NP-treated and untreated cells were exposed to a dose range of 400 mM to 800 mM H₂O₂, for a further 24 hours. To determine the effect of glucose concentration, cells in either 5 mmol/L, or 30 mmol/L glucose were treated for 24 hours, with a dose range of 300 mmol/L to 900 mmol/L H₂O₂.

To assess the effect of dexamethasone, cells were treated with a dose range of 10⁻¹¹ M to 10⁻⁴ mol/L dexamethasone for 24 hours, prior to exposure to 500 mM H₂O₂, for a further 24 hours. Cells were treated with 25, 50, 75, 100, 150 or 200 ng/ml TNF-α to determine the dose required to induce maximal apoptosis. Cells were treated with a dose range of 10⁻¹¹ mol/L to 10⁻⁴ mol/L dexamethasone, for 24 hours and subsequently with 75 ng/ml TNF-α, for a further 24 hours.

Statistical analysis

All values are presented as mean ± SEM in figures and mean (±SD) in text. Student’s t-test was used to determine the significance of differences between the two groups. A P value of 0.05 or less was considered to be statistically significant.

Results

In cells treated with H₂O₂, apoptotic cell death (Fig. 1A) increased as H₂O₂ dose increased, reaching a maximum
at 600 mmol/L H₂O₂. At higher concentrations, the extent of apoptosis decreased sharply, to the level in untreated cells. In contrast, necrosis (Fig. 1 B) increased gradually from the control level at between 300 mmol/L and 600 mmol/L H₂O₂, and more sharply at the higher concentrations. These findings were confirmed by morphological observations of the cells, using phase-contrast microscopy. The degree of viability (Fig. 1 C) remained constant at H₂O₂ doses up to 600 mmol/L, above which viability fell sharply, corresponding to the increase in necrosis.

Pre-treatment with the antioxidant NAC significantly decreased both background levels of apoptosis and H₂O₂-induced apoptosis at H₂O₂ concentrations of 400 mmol/L to 600 mmol/L (P<0.02 at 400 mmol/L and P<0.01 at 500 mmol/L and 600 mmol/L), but apoptosis was increased at higher concentrations of H₂O₂ (800 mmol/L and 900 mmol/L H₂O₂ (P<0.01)) i.e. shifting the peak of apoptosis to the right (Fig. 1 A). In contrast, the extent of necrotic death was significantly higher in the NAC pre-treated cells up to 400 mmol/L H₂O₂ (P<0.05 at 300 mmol/L and P<0.01 at 400 mmol/L) and significantly lower at 800 mmol/L and 900 mmol/L H₂O₂ (P<0.001) (Fig. 1 B). Despite these findings, there was a trend for cell viability to be increased in the presence of NAC, with significance at all levels except 400 mmol/L and 600 mmol/L (Fig. 1 C).

Pre-treatment of EAhy 926 cells with 100 mmol/L deferoxamine significantly reduced apoptosis compared with control at 500 mmol/L (P<0.02) and 600 mmol/L (P<0.01) H₂O₂, but increased it at 800 mmol/L H₂O₂ (p<0.02). At 900 mmol/L H₂O₂, extent of apoptosis was the same, in the presence and absence of deferoxamine (Fig. 1 A). The addition of deferoxamine had no significant effect on the extent of necrosis (Fig. 1 B), except at 800 mmol/L and 900 mmol/L H₂O₂ (P<0.02), at which necrosis was lower than in cells without deferoxamine. Deferoxamine maintained viability of cells (Fig. 1 C) over the range of H₂O₂ concentrations (P<0.01 at 700 mmol/L and P<0.001 at 800 mmol/L and 900 mmol/L, compared with H₂O₂ alone).

Pre-treatment with 3-NP abrogated the apoptotic effect of H₂O₂ at 400 mM (p<0.02) and 500 mM (p<0.01) H₂O₂ (Fig. 2), whilst 3-NP pre-treatment increased necrosis and reduced cell viability (data not shown). The extent of apoptosis (Fig. 3) in cells in 30 mM glucose conditions was higher than in 5 mM glucose, from 500 mM to 800 mM H₂O₂, though differed significantly at 600 mM (p<0.02) alone. Necrosis and cell viability were unaffected by the increase in glucose concentration, exhibiting the same response as shown in Fig. 1 B and 1 C to H₂O₂ alone.

Pre-treatment with dex reduced apoptosis induced at 500 mmol/L H₂O₂, at dex concentrations of 10⁴ mol/L and greater, at both 5 mmol/L and 30 mmol/L glucose (Fig. 4). However, 10⁵ mol/L dex was more effective at reducing apoptosis in 30 mmol/L glucose (P<0.05), whereas 10⁶ mol/L dex lowered the extent of 500 mmol/L H₂O₂-induced necrosis, irrespective of glucose concentration.

In a dose response curve, 100 ng/ml TNF-α induced the maximum increase in apoptosis above the control (untreated) level, in both 5 mmol/L or 30 mmol/L glucose (data not shown). Therefore, 75 ng/ml TNF-α was used in the subsequent experiment.

Pre-treatment with dex at concentrations of 10⁴ mol/L and above, reduced the extent of apoptotic death induced by TNF-α, in both 5 mmol/L and 30 mmol/L glucose concentrations (Fig. 5). The extent of DNA fragmentation was significantly lower in 30 mmol/L glucose, than in 5 mmol/L glucose.

**FIG. 1** Effects of N-acetyl cysteine or deferoxamine, on H₂O₂-induced apoptosis and necrosis, and on cell viability. Cells were exposed to a dose range of 300 mmol/L to 900 μmol/L H₂O₂ alone (closed squares). Treated cells were exposed to either 1 mM N-acetyl cysteine (open squares) or 100 mmol/L deferoxamine (triangles) for 30 minutes prior to the H₂O₂ dose. Assays were performed after 24 hours. Mean ± SEM. n = 3 for each treatment.
glucose, at dext doses of $10^{-11}$ M ($P<0.01$) and $10^{-8}$ M ($P<0.02$).

**Discussion**

This study has shown that exposure to a short term high glucose concentration augments the effects of stress on endothelial cells, suggesting a mechanism by which acute hyperglycemia may be detrimental. These *in vitro* studies are consistent with the effects seen clinically, with the control of acute hyperglycemia in myocardial infarction leading to improved mortality.

Apoptosis was increased in cells treated under oxidative stress in 30 mmol/L glucose, but high glucose alone did not increase apoptosis, suggesting that hyperglycemia was acting as an additional factor in a compromised system. High glucose has been reported to deplete NADPH, inhibiting the activity of the NADPH-requiring antioxidant enzyme, glutathione peroxidase, which is responsible for...
elimination of H$_2$O$_2$ in the cytoplasm, and also to impair cellular radical scavenger function. Therefore, inhibition of synthesis or depletion of endogenous cellular antioxidant defenSes by hyperglycemia, would facilitate additional stress to cause apoptosis. There was no observed effect of 30mM glucose on necrosis, suggesting that the subtle effect of high glucose was lost in the catastrophic process of necrosis.

Cells cultured in high glucose have been reported to exhibit delayed replication, but microscopic observation of cell confluence during the course of this study, revealed no difference in proliferation between 5 mmol/L and 30 mmol/L glucose. This may be due to the comparatively short duration of these experiments (cells were subjected to a maximum of 72 hours in high glucose, prior to assay), as others have reported that the delayed replication seen may only occur after 7 to 14 days of culture in high glucose. Thus, the effects observed here were not due to differences in cell numbers between high and low glucose concentrations.

Whether high-glucose-induced apoptosis has a beneficial or harmful effect on the vasculature is unclear. Baumgartner-Parzer et al. suggested that apoptosis represents one means by which hyperglycemia induced endothelial damage, as a starting point for further deterioration. In contrast, Du et al. propose that endothelial cell apoptosis could be protective, eliminating damaged cells to prevent the accumulation of vascular defects, thus maintaining functional integrity and actually delaying the development of vascular complications.

It was seen that at lower levels of H$_2$O$_2$-induced oxidative stress cells underwent apoptosis, but as the oxidant levels increased, necrosis supervened and apoptosis decreased. Conversely, TNF-α only increased apoptosis to a plateau, without affecting necrosis, suggesting that the two stresses were initiating apoptosis in different ways. However, only H$_2$O$_2$-induced apoptosis was augmented by high glucose levels. The reduction of H$_2$O$_2$-induced apoptosis by the antioxidant, NAC, and the iron chelator, deferoxamine, indicate that the deleterious effects of H$_2$O$_2$ were mediated via the formation of oxidants in this model system. Use of H$_2$O$_2$ in an in vitro model of oxidant injury has previously been evaluated by Li and Lau, in bovine pulmonary artery endothelial cells. The results of the present study are in accord with their findings, that this is an appropriate in vitro model for the endothelium. The attenuation of endothelial cell apoptosis, but not necrosis, by the mitochondrial toxin, 3-NP, confirmed that apoptosis was being measured and that the process was energy-dependent. The apoptotic process requires functional mitochondria, and thus, that ATP-depleted cells are more susceptible to necrosis. Rapid necrotic cell death induced by 3-NP has previously been reported by Pang and Geddes.

The higher level of necrosis seen with NAC treatment was surprising. This may be a result of higher cell numbers and turnover of cells, resulting from antioxidant protection from background levels of oxidative stress. The observed increase in viability is an apparent contradiction of the increased necrosis, but may be due to the fact that the LDH (necrosis) assay is more sensitive than the MTT (viability) assay. The higher viability of cells treated with NAC in the absence of H$_2$O$_2$, indicates that NAC decreases basal levels of cell death and maintains the maximum rate of growth. In the presence of H$_2$O$_2$, growth was decreased, because NAC does not ablate the effects of all types of reactive oxygen species.

Dex reduced apoptosis in both 5 mmol/L and 30 mmol/L glucose. These findings on the effects of both H$_2$O$_2$ and TNF-α are in accord with others reporting glucocorticoids enhancing cell survival under stressful and damaging conditions (Pagliacci et al. 1993, Urayama et al. 1998, Liles et al. 1995). However, dex was more effective at protecting cells from apoptosis in 30 mmol/L glucose than in 5 mmol/L glucose, suggesting that in high glucose cells may be primed to undergo apoptosis more readily.

In conclusion, this in vitro model system has shown that the effect of high glucose concentrations is subtle and not a direct effect on apoptosis or necrosis; however, with an additional stress, apoptosis is increased, though necrosis is unaffected. These data suggest a mechanism by which transient, though recurrent hyperglycemia may induce vascular endothelial damage in vivo.

References

8. Baumgartner-Parzer SM, Wagner L, Pettermann M, et al. Hig-


